UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

**GABRIEL ADAN ARAUJO LEITE**

# “EXPOSIÇÃO DE RATOS MACHOS À ROSUVASTATINA NA PRÉ-PUBERDADE, NA AUSÊNCIA OU PRESENÇA DE VITAMINA C: EFEITOS IMEDIATOS E TARDIOS SOBRE O SISTEMA GENITAL E A FERTILIDADE DAS GERAÇÕES F0 E F1”

# “MALE RATS EXPOSED TO ROSUVASTATIN DURING PRE-PUBERTY IN THE ABSENCE OR PRESENCE OF VITAMIN C: SHORT- AND LONG-TERM EFFECTS ON THE GENITAL SYSTEM AND THE FERTILITY OF GENERATIONS F0 AND F1”

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**2018**

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*Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.*

*Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Cellular and Structural Biology, in the area of Cell Biology.*

Orientadora: PROFª. DRª. WILMA DE GRAVA KEMPINAS

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO GABRIEL ADAN ARAUJO LEITE, E ORIENTADA PELA PROFA. DRA. WILMA DE GRAVA KEMPINAS

CAMPINAS

2018

**Agência(s) de fomento e nº(s) de processo(s):** FAPESP, 2013/22495-1; CAPES
**ORCID:** https://orcid.org/0000-0002-9437-6310

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

L536e

Leite, Gabriel Adan Araujo, 1990-
Exposição de ratos machos à rosuvastatina na pré-puberdade, na ausência ou presença de vitamina C : efeitos imediatos e tardios sobre o sistema genital e a fertilidade das gerações F0 e F1 / Gabriel Adan Araujo Leite. – Campinas, SP : [s.n.], 2018.

Orientador: Wilma De Grava Kempinas.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Rosuvastatina cálcica. 2. Vitamina C. 3. Rato - Reprodução. 4. Toxicologia. 5. Fertilidade. I. Kempinas, Wilma De Grava. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

**Título em outro idioma:** Male rats exposed to rosuvastatin during pre-puberty in the absence or presence of vitamin C : short- and long-term effects on the genital system and the fertility of generations F0 and F1
**Palavras-chave em inglês:**
Rosuvastatin calcium
Vitamin C
Rats - Reproduction
Toxicology
Fertility
**Área de concentração:** Biologia Celular
**Titulação:** Doutor em Biologia Celular e Estrutural
**Banca examinadora:**
Wilma De Grava Kempinas [Orientador]
Patricia Aline Boer
Mary Anne Heidi Dolder
Arielle Cristina Arena
Camila Contin Diniz de Almeida Francia
**Data de defesa:** 02-04-2018
**Programa de Pós-Graduação:** Biologia Celular e Estrutural

Campinas, 02 de abril de 2018.

**COMISSÃO EXAMINADORA**

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**Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.**

Dedico este trabalho aos meus pais, Vera e Milton, por todo amor, carinho e incentivo inestimáveis que recebi. Obrigado por todos os ensinamentos que me deram e por terem sido meu amparo em todos os momentos que precisei.

*"O amor verdadeiro começa lá onde não se espera mais nada em troca."*

*Antoine de Saint-Exupéry*

## AGRADECIMENTOS

A Deus, pela vida, por ter iluminado meu caminho e por ter me capacitado para que esta etapa pudesse ser concluída.

Aos meus pais, Milton e Vera, minha irmã, Jéssica, e minha avó, Yolanda (*in memoriam*), por sempre terem me incentivado, e apesar de estarem distantes fisicamente, por sempre terem me dado forças para continuar e concluir meus sonhos e objetivos.

À minha orientadora, Profa. Dra. Wilma De Grava Kempinas, pela amizade, por ter acreditado e confiado em mim para a realização desse sonho, por tudo que me ensinou e por despertar em mim o amor pela docência e pela pesquisa.

Ao Laboratório ReproTox, por toda ajuda recebida e todo aprendizado adquirido ao longo desses anos, porque sem a ajuda de todos, esse estudo não teria sido possível.

À Thamiris e a Tainá, alunas de iniciação científica na época, que foram muito importantes para o desenvolvimento desse projeto.

Às amigas que o Laboratório ReproTox me deu, Marina e Marci, que tornaram minha caminhada bem mais leve.

Aos animais experimentais, porque foram essenciais para a realização deste estudo.

Aos amigos que a vida me deu, Danilo, Douglas e Vinícius, que acompanharam de perto a realização desse sonho.

A todos os professores, porque de alguma maneira contribuíram para a pessoa e profissional que sou hoje.

Ao Programa de Pós-Graduação em Biologia Celular e Estrutural, Instituto de Biologia, Unicamp.

À secretária do Programa de Pós-graduação em Biologia Celular e Estrutural, Liliam, por toda ajuda cedida e por sempre estar disposta a ajudar.

Ao Departamento de Morfologia do Instituto de Biociências de Botucatu (UNESP), pelo espaço físico que possibilitou a realização deste estudo.

À secretária do departamento de Morfologia, Luciana, por toda ajuda concedida.

À Dra. Janete Aparecida Anselmo-Franci e ao Dr. Ruither de Oliveira Gomes Carolino do Laboratório de Estomatologia e Fisiologia da USP - Ribeirão Preto, pela realização das dosagens hormonais.

Ao Dr. Fernando Barbosa Junior e ao Airton da Cunha Martins Junior da Faculdade de Ciências Farmacêuticas de Ribeirão Preto da USP, pela colaboração nas análises da atividade antioxidante e de estresse oxidativo.

Ao José Eduardo Bozano, pela assistência e ajuda sempre, e acima de tudo, pela sincera amizade.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Processo Fapesp: 2013/22495-1) pelo auxílio financeiro na forma de bolsa para a realização deste trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo auxílio financeiro na forma de bolsa nos primeiros meses da realização deste estudo.

A todos que de alguma maneira contribuíram para que esse estudo se tornasse possível.

**RESUMO**

A obesidade está afetando crianças e adolescentes em diversos países. Tem sido relatado que as disfunções no perfil lipídico estão associadas com a obesidade, o estilo de vida inapropriado e o sedentarismo. A rosuvastatina é um medicamento de última geração que diminui os níveis séricos colesterol e triglicérides. O ácido ascórbico é um importante composto antioxidante que é essencial para a fertilidade. Os objetivos do estudo foram investigar os parâmetros reprodutivos e a fertilidade das gerações F0 e F1 de ratos expostos à rosuvastatina e avaliar o papel do ácido ascórbico na prevenção desses efeitos adversos. Ratos machos pré-puberes foram distribuídos em seis grupos experimentais que receberam solução salina 0,9% (veículo), 3 ou 10 mg/Kg/dia de rosuvastatina, 150 mg/dia de ácido ascórbico, ou 3 ou 10 mg/Kg/dia de rosuvastatina co-administrada com 150 mg/dia de ácido ascórbico por gavagem do dia pós-natal (DPN) 23 até o DPN 53. Os ratos (n=10/grupo) foram eutanasiados no DPN 53 para avaliação dos efeitos reprodutivos imediatos. Os demais ratos (n=10/grupo) foram mantidos até a maturidade sexual (DPN 100) quando o comportamento sexual e a fertilidade dos machos foram avaliadas. Os ratos foram acasalados com mais de uma fêmea para obtenção da sua prole masculina e feminina. No DPN 110, os animais controle e tratados (n=10/grupo) foram eutanasiados e os parâmetros reprodutivos foram avaliados. A prole feminina foi avaliada na puberdade e na maturidade sexual, e a prole masculina foi estudada na vida adulta em relação aos parâmetros reprodutivos. Os grupos expostos à rosuvastatina mostraram atraso na instalação da puberdade, depleção androgênica e danos na morfologia testicular e epididimária na puberdade. Além disso, esses grupos apresentaram menor qualidade espermática, menores concentrações de testosterona, menor marcação para os receptores androgênicos nucleares nas células de Sertoli nos estágios IX-XIII, aumento na frequência da morte de células germinativas e maior estresse oxidativo na maturidade sexual. A prole masculina dos grupos tratados com a rosuvastatina exibiram aumento da fragmentação do DNA, depleção androgênica, aumento na taxa de morte de células germinativas e prejuízo para a histoarquitetura epididimária. A prole feminina dos pais que foram expostos ou co-expostos à maior dose da estatina apresentou menor número de corpos lúteos na puberdade. Na maturidade sexual, as fêmeas do grupo cujos pais foram expostos à dose de 3 mg de rosuvastatina demonstraram menor área do epitélio luminal uterino. A co-administração de ácido ascórbico para ratos machos pré-púberes diminuiu o dano reprodutivo nas gerações $F_0$ e $F_1$ promovidos pelo tratamento com a rosuvastatina. Em resumo, a exposição pré-puberal à

rosuvastatina afetou a reprodução nas gerações $F_0$ e $F_1$, provavelmente devido a depleção androgênica, aumento no estresse oxidativo, presença de maiores danos no DNA espermático e as possíveis alterações epigenéticas no espermatozoide dos pais. Entretanto, o ácido ascórbico foi capaz de estimular a esteroidogênese, reduzir o estresse oxidativo e melhorar a capacidade antioxidante, além de proteger, pelo menos parcialmente, o espermatozoide dos pais de possíveis alterações epigenéticas, diminuindo dessa forma, os danos reprodutivos promovidos nas gerações $F_0$ e $F_1$ pelo tratamento com a rosuvastatina.

**ABSTRACT**

Obesity is affecting children and adolescents in several countries. It is known that dysfunctions on lipid profile are related to obesity, inappropriate lifestyle and sedentary habits. Rosuvastatin is a first-line therapy medication that decreases serum cholesterol and triglycerides levels. Ascorbic acid is an important antioxidant compound that is essential for fertility and sperm integrity. The study aimed to investigate the reproductive parameters and fertility of generations $F_0$ e $F_1$ of male rats exposed to rosuvastatin and evaluate the role of ascorbic acid preventing these adverse effects. Pre-pubertal male rats were distributed into six experimental groups that received saline solution 0.9% (vehicle) , 3 or 10 mg/Kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 3 or 10 mg/Kg/day of rosuvastatin co-administered with 150 mg/day of ascorbic acid by gavage from post-natal day (PND) 23 until PND 53. Male rats (n=10/group) were euthanized on PND 53 to assess the short-term reproductive effects of rosuvastatin exposure and the possible preventive role of ascorbic acid in reproduction. The remaining rats (n=10/group) were maintained until sexual maturity (PND 100) when sexual behavior test and fertility were evaluated. Males were mated with an additional non-treated female rat to obtain their male and female offspring. On PND 110, the control and treated males (n=10/group) were euthanized and the reproductive parameters were assessed. Female offspring were evaluated at puberty and adulthood, and male offspring were evaluated on sexual maturity in relation to their reproductive parameters. Rosuvastatin-treated groups showed delayed puberty installation, androgen depletion and impairment on testicular and epididymal morphology at puberty. Additionally, they showed lower sperm quality, decreased testosterone concentrations, weaker androgen receptors nuclear staining in Sertoli cells at stages IX-XIII, increased germ cell death and augmented oxidative stress at adulthood. Male offspring from rosuvastatin-exposed groups showed increased sperm DNA fragmentation, androgen depletion, an augmented rate of germ cell death and impairment on the epididymal structure. Female offspring whose fathers were exposed or co-exposed to the higher dose of statin showed a lower number of corpora lutea during puberty. On sexual maturity, females from the group whose fathers were exposed to 3 mg showed lower uterine luminal epithelium area. Ascorbic acid co-administered to pre-pubertal male rats ameliorated the reproductive damage in the $F_0$ and $F_1$ generation promoted by rosuvastatin exposure. In summary, pre-pubertal exposure to rosuvastatin impaired the reproduction in the $F_0$ and $F_1$ generation, probably due to the androgen depletion, increased oxidative stress, augmented sperm DNA

damage and the possible epigenetic changes in paternal sperm. On the other hand, ascorbic acid was able to stimulate steroidogenesis, reduce oxidative stress, and improve antioxidant status, besides to protect, at least partially, paternal sperm from the possible epigenetic changes, thus alleviating the reproductive damage in the $F_0$ and $F_1$ generation promoted by rosuvastatin exposure.

## SUMÁRIO

# 1. INTRODUÇÃO

## 1.1. Obesidade, dislipidemias e agentes hipolipemiantes

A obesidade é um dos grandes problemas emergentes de saúde pública que tem afetado adultos, crianças e adolescentes, e se apresenta como o transtorno metabólico e nutricional com maior destaque nos países desenvolvidos, afetando também os países subdesenvolvidos (Jiménez e Ferre, 2011).

Em consequência do aumento do número de obesos, as dislipidemias têm sido um achado frequente e se manifestado cada vez mais cedo na população, sendo ainda mais comum na faixa pediátrica, principalmente em crianças e adolescentes que apresentam obesidade variando de leve a moderada, o que acarreta em níveis aumentados de LDL-colesterol e VLDL-colesterol, diminuição do HDL-colesterol sérico (Jiménez e Ferre, 2011) e aumento dos níveis séricos de triglicerídeos (Kwiterovich, 2008).

A obesidade pediátrica, além da íntima relação com as dislipidemias, tem sido relacionada com o risco aumentado de doenças cardiovasculares, resistência à insulina, hipertensão, aceleração do processo aterosclerótico (McGill *et al.*, 2002; Klop *et al.*, 2013), diabetes mellitus do tipo 2, síndrome metabólica, disfunções reprodutivas como a síndrome do ovário policístico, problemas associados ao sono e ao sistema gastrointestinal, e alterações psicológicas, incluindo a depressão (Seth e Sharma, 2013).

As dislipidemias podem ser desencadeadas na infância ou mais tardiamente devido ao estilo de vida inapropriado, que incluem o sedentarismo, a má alimentação e outros fatores relacionados ao estilo de vida (Izar *et al.*, 2011).

A prevalência das hipercolesterolemias em crianças e adolescentes varia no mundo entre 2,9 e 33%, quando considerados os níveis séricos de colesterol total acima de 200 mg/dL (Giuliano e Caramelli, 2008). No Brasil, o panorama das hipercolesterolemias em crianças e adolescentes está entre 28 e 40%, considerando que níveis de colesterol excedendo os 170 mg/dL têm sido o critério adotado para determinação de hipercolesterolemia infantil (Giuliano e Caramelli, 2008).

Em crianças e adolescentes os níveis de colesterol total devem ser menores do que 170 mg/dL, o LDL-colesterol menor que 110 mg/dL, o HDL-colesterol maior que 45 mg/dL e os triglicerídeos séricos devem ser menores que 75 mg/dL para crianças com até 9 anos de idade e menores que 90 mg/dL para crianças a partir de 10 anos até o começo da idade adulta, ou seja, até os 19 anos de idade (Cook e Kavey, 2011).

Nos EUA, o tratamento medicamentoso para as dislipidemias segue as normas da Academia Americana de Pediatria que recomenda que o uso de agentes hipolipemiantes seja destinado a crianças e adolescentes que apresentam LDL-colesterol maior que 190 mg/dL, desde que não haja outros fatores de risco associados (Cook e Kavey, 2011).

Em caso de fatores de risco como obesidade, hipertensão, fumo ou histórico de doença cardiovascular familiar, o uso de medicamentos é recomendado a partir de 160 mg/dL de LDL-colesterol sérico, desde que se apresente pelo menos dois desses fatores de risco (Cook e Kavey, 2011). No caso de pacientes que apresentam diabetes mellitus, o nível sérico de LDL-colesterol máximo permitido é de 130 mg/dL antes de se partir para o tratamento hipolipemiante (Cook e Kavey, 2011).

O tratamento farmacológico para as dislipidemias pode ser realizado através das seguintes classes de medicamentos, com diferentes mecanismos de ação: estatinas, inibidores de absorção intestinal do colesterol, ácido nicotínico, ácidos graxos ômega-3 e os fibratos, derivados do ácido fíbrico (Izar *et al.*, 2011), sendo que destes, as estatinas são os fármacos que tem maior destaque (Jiménez e Ferre, 2011).

As estatinas são inibidores da enzima 3-hidroxi-3-metilglutaril coenzima A redutase (HMG-CoA redutase) que é uma enzima limitante para a biossíntese do colesterol (Istvan and Deisenhofer, 2001; Jiménez and Ferre, 2011), e portanto, as estatinas são responsáveis por diminuir o colesterol total, reduzindo principalmente o LDL-colesterol (Istvan e Deisenhofer, 2001; Tandon *et al.*, 2005; Endres, 2006).

As estatinas ao inibirem a enzima HMG-CoA redutase impedem a conversão dO HMG-CoA em mevalonato, e consequentemente diminuem a formação do colesterol endógeno (Figura 1). Estes fármacos também inibem a síntese de outros compostos importantes, tais como os isoprenóides intermediários, dos quais se destacam o geranilgeranilpirofosfato (GGPP) e farnesilpirofosfato (FPP) (Figura 1) (Istvan, 2003; Adam e Laufs, 2008).

Estão disponíveis para comercialização no Brasil seis estatinas: fluvastatina, lovastatina, sinvastatina, atorvastatina, pravastatina e rosuvastatina (Figura 2) (Sociedade Brasileira de Cardiologia, 2007). Estes fármacos diferem entre si com relação às suas propriedades farmacológicas, tais como: afinidade pelo sítio ativo da enzima HMG-CoA redutase, taxa de entrada nos hepatócitos e em outros tecidos, disponibilidade do fármaco na circulação sistêmica para captação por tecidos extra-hepáticos e mecanismos de transformação metabólica (McTaggart, 2003).

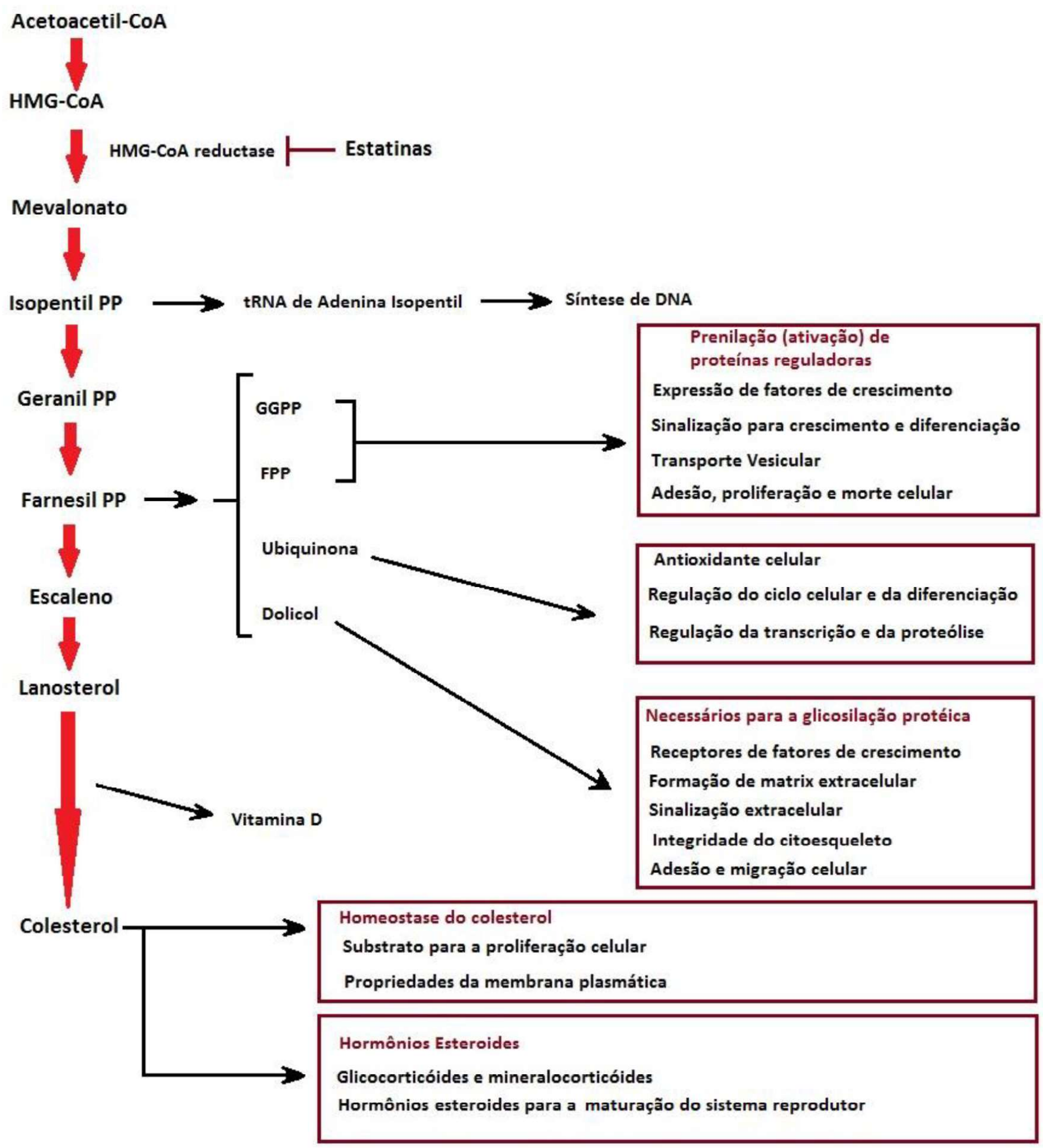


**Figura 1.** Esquema do mecanismo de ação das estatinas e da importância dos compostos da via do mevalonato para as diferentes funções biológicas. Imagem adaptada (Edison e Muenke, 2004).

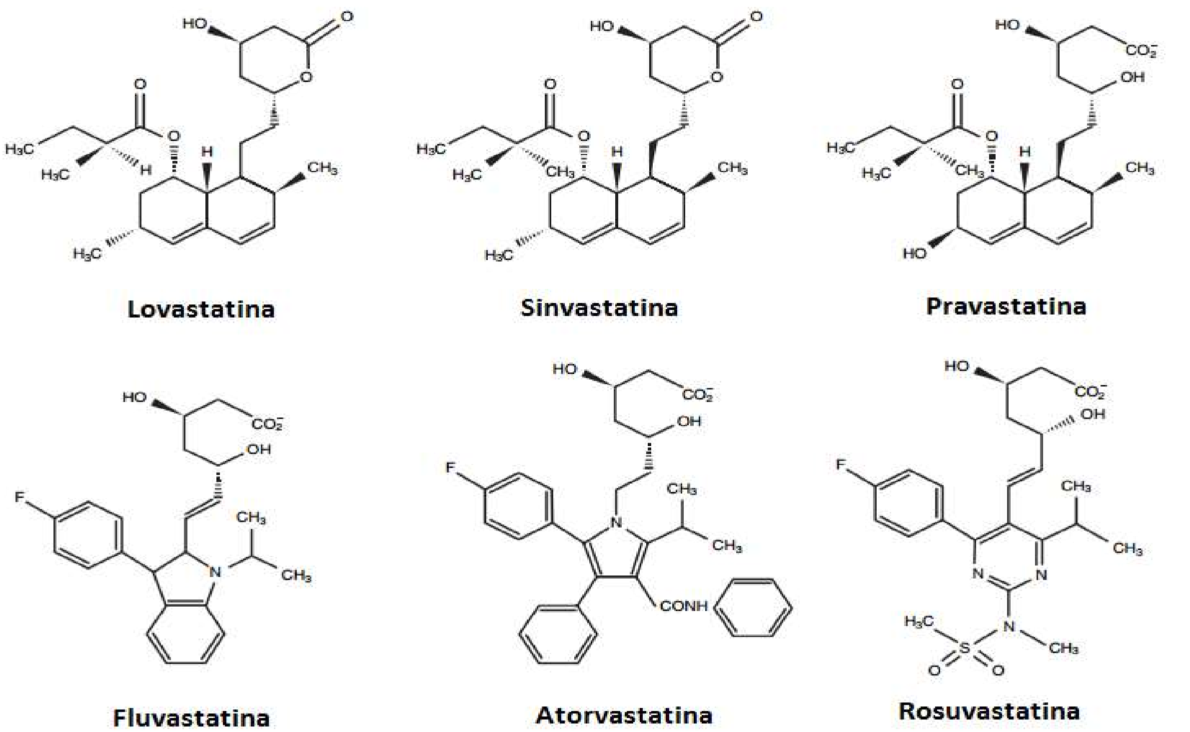


**Figura 2.** Estrutura química das estatinas disponíveis para comercialização no Brasil. Imagem adaptada (Schachter, 2005).

Os inibidores da HMG-CoA redutase têm sido indicados como potenciais anti-inflamatórios, imunomoduladores e protetores vasculares para o tratamento de doenças cerebrais como a doença de Alzheimer, esclerose múltipla e depressão (Corder *et al.*, 1993; Campo e Carvalho, 2007), além de possuírem potencial para o tratamento da osteoporose, devido aos seus efeitos osteogênicos (Campo e Carvalho, 2007).

Vários estudos têm demonstrado o grande potencial de inibição de proliferação e a ação de indução da morte celular em linhagens celulares normais e tumorais promovidos pelas estatinas (Feleszko *et al.*, 1998; Tanaka *et al.*, 2000; Lee e Kim, 2013; Qi *et al.*, 2013). Os efeitos antineoplásicos promovidos pelas estatinas em muitos tipos de tumores ocorre através da indução da morte celular e da parada do ciclo celular de maneira independente da diminuição do colesterol, sendo esses efeitos mais associados à diminuição dos isoprenóides intermediários (Lee e Kim, 2013; Qi *et al.*, 2013).

Em culturas celulares de linfoma, as estatinas exibiram aumento na fragmentação do DNA, ativação de membros pró-apoptóticos (caspases-3 e Bax), suprimiram a ação da molécula anti-apoptótica Bcl-2 e aumentaram as espécies reativas de oxigênio em decorrência da inibição de compostos da via do mevalonato, tais como o GGPP, FPP e o próprio mevalonato (Qi *et al.*, 2013).

Em comparação com as outras estatinas comercializadas no Brasil, a rosuvastatina (rosuvastatina cálcica) apresenta várias vantagens farmacológicas, dispondo de características únicas de ligação com a enzima HMG-CoA redutase e efeitos inibitórios superiores em relação às outras estatinas (Holdgate *et al.*, 2003; McTaggart, 2003). Além disso, este medicamento é altamente hidrofílico e apresenta seletividade por hepatócitos, o que sugere menor toxicidade para as fibras musculares esqueléticas devido ao baixo potencial de inibição do colesterol nestes tipos de fibras musculares, uma vez que alterações musculares têm sido relatadas como o principal efeito adverso das estatinas (McTaggart, 2003).

A rosuvastatina é a estatina que mais reduz os níveis séricos de LDL-colesterol (Fonseca, 2005) e os níveis de colesterol total quando comparada com as outras estatinas na mesma dose utilizada diariamente, indicando que para a dose de 10 mg/dia ocorre diminuição de 43% do colesterol total e para a dose de 40 mg/dia os níveis de colesterol total alcançam uma redução de 46% a 53% (Vaughan e Gotto Jr, 2004; Penning-Van Beest *et al.*, 2007).

Considerando as estatinas disponíveis no mercado, a rosuvastatina é a estatina que possui maior tempo de meia-vida para ser eliminada (20 horas), quando comparado com a atorvastatina (15 horas), pravastatina, fluvastatina e sinvastatina (1-3 horas) (McTaggart, 2003).

A rosuvastatina tem demonstrado capacidade similar na redução dos níveis de LDL-colesterol quando administrada em diferentes períodos do dia, diferentemente das outras estatinas, ou seja, a farmacocinética da rosuvastatina independe do seu horário de administração, e esta característica está diretamente relacionada com o seu tempo de meia-vida relativamente longo (Martin *et al.*, 2002).

## 1.2. Instalação da puberdade nos machos e desreguladores endócrinos

As estatinas ao reduzirem os níveis séricos de colesterol e os níveis de hormônios esteroides sexuais podem agir como desreguladores endócrinos. A puberdade é um período crítico considerado como uma fase importante para a saúde reprodutiva, pois marca a transição da infância para o estágio reprodutivo adulto, além de ser o momento em que ocorrem rápidas interações neuroendócrinas e alterações morfológicas importantes nos mamíferos (Stoker *et al.*, 2000; Zawatski e Lee, 2013).

A instalação da puberdade se inicia com pulsos mais frequentes de hormônio liberador de gonadotrofina (GnRH) no hipotálamo e com o aumento da secreção de hormônio folículo-estimulante (FSH) e hormônio luteinizante (LH) liberados pela hipófise, levando à estimulação da secreção de hormônios esteroides sexuais pelos testículos e à maturação dos túbulos seminíferos, permitindo consequentemente, a aquisição da capacidade reprodutiva e das características sexuais secundárias (Fisher e Eugster, 2014; Mantovani e Fucic, 2014).

Um fator importante que está relacionado com a ativação dos neurônios hipotalâmicos liberadores de gonadotrofinas (neurônios GnRH) é o aumento da secreção de kisspeptina pelos neurônios hipotalâmicos KISS1, o qual é reconhecido pelos neurônios GnRH, estimulando esses neurônios a secretar o GnRH em pulsos mais frequentes (Figura 3) (Bianco, 2012; Choi e Yoo, 2013).

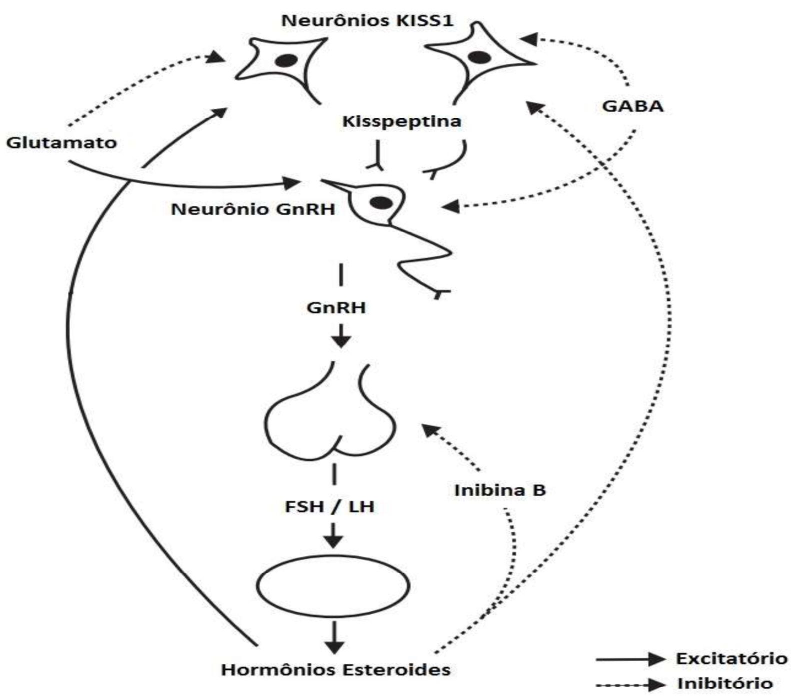


**Figura 3.** Esquema do papel da kisspeptina e do GnRH na ativação do eixo hipotalâmico-hipofisário-gonadal durante a instalação da puberdade. Imagem modificada (Choi e Yoo, 2013).

Os fatores que determinam a ativação do eixo hipotalâmico-hipofisário-gonadal e o aumento na secreção  pulsátil de GnRH ainda não estão bem definidos, porém alguns fatores, tais como o crescimento somático, a gordura corpórea, a dieta, o estresse, o ritmo circadiano, o gasto energético e os estímulos olfatórios têm sido associados com a modulação e secreção de GnRH pelos neurônios GnRH através de influências que exercem na rede neuronal, regulando deste modo, o tempo de instalação da puberdade (Figura 4) (Ebling, 2005).

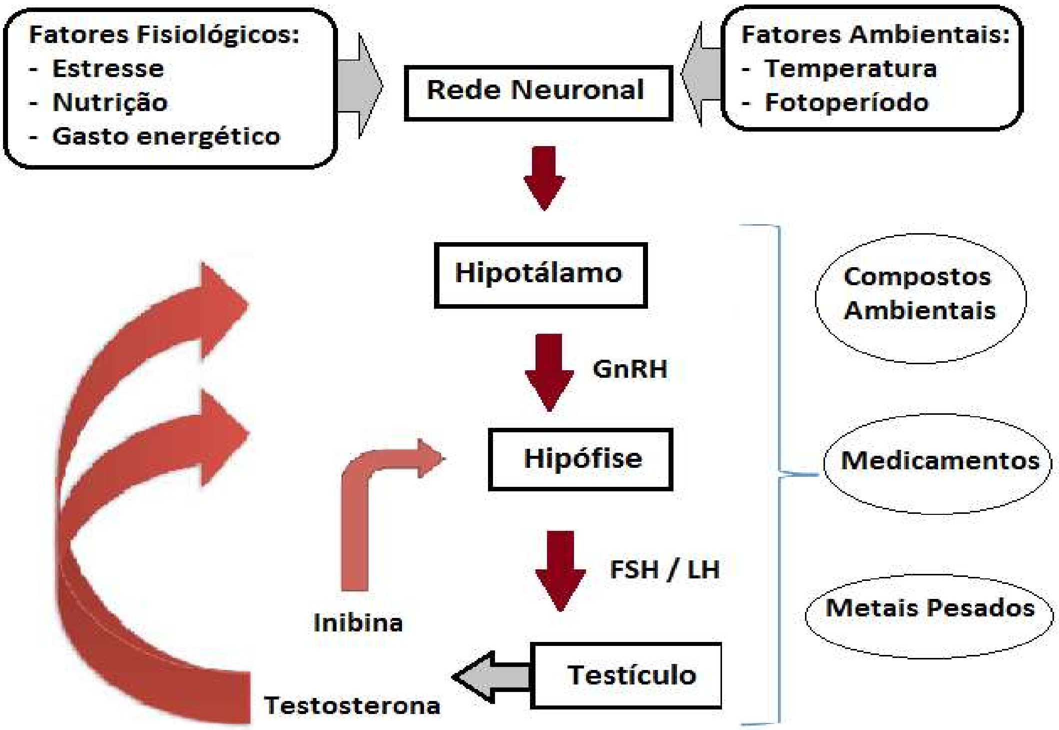


**Figura 4.** Influência de fatores fisiológicos e ambientais na rede neuronal e ativação do eixo hipotalâmico-hipofisário-gonadal. Imagem adaptada (Zawatski e Lee, 2013).

As crianças e adolescentes estão expostos a vários agentes químicos que podem alterar o tempo de instalação da puberdade e o desenvolvimento e aquisição da função reprodutiva, sendo essa problemática de grande importância para a toxicologia reprodutiva (Stoker *et al.*, 2000), uma vez que alterações na puberdade podem inclusive afetar a função reprodutiva na maturidade sexual (Perobelli *et al.*, 2012, 2013; Mantovani e Fucic, 2014).

A exposição a possíveis desreguladores endócrinos na infância e adolescência pode alterar o crescimento e a distribuição da gordura corporal ainda neste período, bem como promover alterações de comportamento e aumentar o risco de câncer na vida adulta (Mantovani e Fucic, 2014).

Os desreguladores endócrinos que tendem a atrasar a instalação da puberdade masculina têm sido associados com a ocorrência de síndrome metabólica, osteoporose e fraturas ósseas na vida adulta (Bianco, 2012). Durante a peripuberdade a espermatogênese e a esteroidogênese ainda não estão completamente estabelecidas, o que torna o sistema genital masculino mais susceptível a ação de agentes químicos (Johnson *et al.*, 1997; Favareto *et al.*, 2011a). Muitas substâncias químicas diminuem a função androgênica e podem atrasar o desenvolvimento do sistema genital masculino, sendo que os efeitos adversos promovidos por esses agentes tóxicos são dependentes do tempo de exposição a esses compostos (Blystone *et al.*, 2007).

## 1.3. O papel do ácido ascórbico no organismo

Atualmente, muitos estudos têm buscado diminuir ou reverter os efeitos adversos promovidos por diversas patologias, disfunções metabólicas ou mesmo por agentes químicos utilizados como importantes medicamentos em vários tratamentos (Fernandes *et al.*, 2011a, b; Mukhopadhyay *et al.*, 2013). A administração de um composto que apresente potencial de ação antioxidante ou outro mecanismo que possa prevenir a ação tóxica de um determinado agente químico tem sido amplamente investigada (Corsetti *et al.*, 2011; Sooriyaarachchi *et al.*, 2012; Mukhopadhyay *et al.*, 2013; Pandir *et al.*, 2014), uma vez que muitos medicamentos precisam ser utilizados para o tratamento de várias doenças e disfunções metabólicas (Sooriyaarachchi *et al.*, 2012; Pandir *et al.*, 2014).

O ácido ascórbico (vitamina C) está presente em vários alimentos, dos quais se destacam as frutas cítricas, tais como a laranja e o limão, além de outras frutas, como a banana e o morango (Okon e Utuk, 2016). Vários legumes apresentam níveis relevantes de ácido ascórbico, os quais incluem o brócolis, o repolho e a couve-flor (Okon e Utuk, 2016).

O ácido ascórbico é uma substância hidrossolúvel necessária para muitas reações bioquímicas no organismo e apresenta várias funções biológicas, as quais incluem o seu papel como nutriente essencial para a biossíntese do colágeno e da noradrenalina, e para a reciclagem do α-tocoferol (Sönmez *et al.*, 2005; Fernandes *et al.*, 2011a, b). Além disso, o ácido ascórbico participa como importante agente redutor em muitas reações intra e extracelulares, atua como cofator enzimático para enzimas dependentes de cobre e ferro, e está envolvido na formação da tirosina (German Nutrition Society, 2015). A estrutura química da molécula do ácido ascórbico é mostrada na Figura 5.

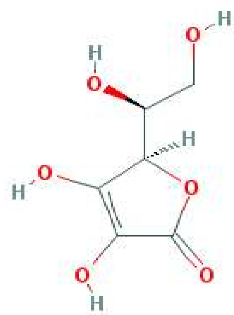


**Figura 5.** Estrutura química da molécula do ácido ascórbico. Imagem obtida do Pubchem (Open Chemistry Database).

A vitamina C é uma substância de grande capacidade antioxidante que elimina radicais livres e outras espécies reativas de oxigênio e nitrogênio, como os radicais superóxido e hidroxila, além do ácido hipocloroso (German Nutrition Society, 2015). Deste modo, a vitamina C protege as biomembranas contra a peroxidação lipídica e protege o DNA contra o dano oxidativo (German Nutrition Society, 2015). Ademais, alguns estudos têm demonstrado o papel protetor da vitamina C na oxidação do HDL-colesterol (Hillstrom *et al.*, 2003; Siavash e Amini, 2014) e relatado aumento do HDL-colesterol devido a suplementação com a vitamina C (Yanai e Morimoto, 2004; Siavash e Amini, 2014).

No sistema reprodutor masculino, o ácido ascórbico exerce importante função na integridade espermática e na fertilidade, atua como protetor para a espermatogênese e como agente antioxidante contra o estresse oxidativo testicular (Agarwal *et al.*, 2005; Eskenazi *et al.*, 2005; Shrilatha e Muralidhara, 2007; Fernandes *et al.*, 2011b). Além disso, desempenha papel importante na regulação da esteroidogênese testicular e da adrenal, podendo aumentar as concentrações de testosterona (Sönmez *et al.*, 2005). Sabe-se ainda que a vitamina C é encontrada em altas concentrações no fluido epididimário e no sêmen, desempenhando papel importante na prevenção da excessiva peroxidação lipídica (Sönmez *et al.*, 2005).

## 1.4. Atividade antioxidante e estresse oxidativo

Nos mamíferos, o estresse oxidativo está associado com o aumento da produção de espécies reativas de oxigênio e nitrogênio que podem causar modificações no DNA e em proteínas, e consequentemente, aumentar as concentrações de produtos resultantes da peroxidação lipídica, superando assim, a capacidade antioxidante endógena das células e tecidos (Mouthuy *et al.*, 2016).

O sistema antioxidante, pelo menos parcialmente, é formado por pequenas moléculas solúveis em água ou em lipídios, das quais se destacam a glutationa, o ácido ascórbico, os tocoferóis e os tocotrienóis (Mouthuy *et al.*, 2016). Ademais, algumas enzimas também fazem parte do sistema antioxidante, tais como a catalase e a glutationa peroxidase que participam na detoxificação do peróxido de hidrogênio e decompõe essa molécula altamente reativa, gerando como produtos finais a molécula de água ($H_2O$) e o gás oxigênio ($O_2$), (Aebi, 1984; Mouthuy *et al.*, 2016). Através da detoxificação enzimática ocorre uma diminuição na excessiva peroxidação lipídica, a qual evita os danos aos componentes celulares e aos tecidos (Aebi, 1984; Baud, 2004; Mouthuy *et al.*, 2016). Além disso, a enzima catalase tem demonstrado ser a primeira enzima recrutada quando há grandes concentrações de peróxido de hidrogênio (Baud, 2004).

A maturação do sistema antioxidante ocorre com a progressão do desenvolvimento puberal e é acompanhada do aumento das concentrações de testosterona e do hormônio do crescimento (GH) (Paltoglou *et al.*, 2015; Avloniti *et al.*, 2017). O ácido ascórbico, que é um dos componentes importantes do sistema antioxidante, é capaz de neutralizar uma grande quantidade de radicais, tais como o hidroxil, superóxido e o peróxido de hidrogênio, e assim prevenir o estresse oxidativo e atuar na maturação desse sistema (Makker *et al.*, 2009; Paltoglou *et al.*, 2015; Avloniti *et al.*, 2017).

Além disso, sabe-se que a vitamina C é responsável por cerca de até 65% por cento da atividade antioxidante no sêmen, o que demonstra o seu papel antioxidante importante no sistema reprodutor masculino (Makker *et al.*, 2009; Fernandes *et al.*, 2011b).

## 1.5. Estatinas, ácido ascórbico e epigenética

A epigenética é caracterizada por mudanças no padrão de metilação do DNA, modificações nas proteínas histonas, tais como a acetilação, metilação ou ainda remodelamento da cromatina (Rajender *et al.*, 2011). Estes mecanismos epigenéticos são essenciais para muitos processos biológicos, incluindo a espermatogênese, pois afetam a expressão gênica mesmo sem afetar a estrutura do DNA (Rajender *et al.*, 2011).

Vários estudos já sugeriram ou relataram alterações epigenéticas no espermatozoide comprometendo o desenvolvimento embrionário ou causando efeitos adversos na prole, incluindo desordens reprodutivas (Favareto *et al.*, 2011b; Stuppia *et al.*, 2015; Schagdarsurengin e Steger, 2016; Silva *et al.*, 2016; Ly *et al.*, 2017). O aumento no estresse oxidativo tem sido considerado como umas das causas do aumento no dano do DNA espermático e tem sido associado com um padrão global aberrante de metilação espermática (Rajender *et al.*, 2011). Por outro lado, as substâncias antioxidantes têm sido capazes de diminuir a fragmentação do DNA, o estresse oxidativo e o padrão aberrante de metilação global espermático (Rajender *et al.*, 2011).

A exposição paterna ou materna a diferentes substâncias químicas tóxicas tem sido capaz de modificar o epigenoma do gameta e afetar a saúde das suas respectivas proles (Schagdarsurengin e Steger, 2016). As alterações epigenéticas no espermatozoide podem afetar a reprodução masculina levando a falhas na espermatogênese, comprometendo o desenvolvimento embrionário e gerando efeitos adversos a longo prazo em algum momento na vida dos seus descendentes (Stuppia *et al.*, 2015). Desta forma, as alterações epigenéticas podem ter efeitos a curto ou longo prazo, ou podem ainda apresentar efeitos transgeracionais, podendo afetar as gerações subsequentes (Rajender *et al.*, 2011).

A reprogramação epigenética das células germinativas ocorre durante o início do desenvolvimento embrionário e durante a espermatogênese, mas quando ocorre falhas nesse processo, as alterações epigenéticas conseguem se manter e afetar a prole (Heard e Martienssen, 2014). Estudos recentes têm indicado que as estatinas podem induzir alterações epigenéticas, entretanto, a literatura existente ainda é escassa (Allen e Mamotte, 2017; Arrigoni *et al.*, 2017).

A vitamina C, se deficiente no organismo, pode alterar a dinâmica de metilação e desmetilação do DNA e das proteínas histonas, contribuindo para modificações fenotípicas (Camarena e Wang, 2016). Sendo assim, o ácido ascórbico regula o epigenoma e está envolvido no desenvolvimento embrionário, desenvolvimento pós-natal, no envelhecimento e no desencadeamento de doenças (Camarena e Wang, 2016). O ácido ascórbico, conhecido como ascorbato na sua forma intracelular, regula a desmetilação do DNA, uma vez que é um cofator essencial para as enzimas ten-eleven translocation (TET) -desoxigenases, além de regular a desmetilação de proteínas histonas, porque atua como cofator para as enzimas histonas desmetilases com domínio Jumonji (Young et al., 2015).

## 1.6. Diferenciação das gônadas e dos ductos genitais

No início do desenvolvimento embrionário, machos e fêmeas apresentam uma gônada indiferenciada que poderá se diferenciar em testículo ou ovário, e que tem origem a partir da crista genital e das células germinativas primordiais que migraram até o primórdio da gônada (George e Wilson, 1994; Drews, 2000; Griswold e Behringer, 2009). As células germinativas primordiais darão origem às espermatogônias no testículo e originarão as ovogônias nos ovários (Saitou e Yamaji, 2012).

A diferenciação da gônada masculina, o testículo, ocorre primeiro do que a diferenciação da gônada feminina (Jost *et al.*, 1973). O gene SRY que está localizado no cromossomo Y, e portanto somente está presente em indivíduos com sexo cromossômico XY (sexo masculino), é um gene determinante na diferenciação testicular (George e Wilson, 1994; Griswold e Behringer, 2009). A expressão do gene SRY faz com que as células somáticas presentes na crista genital se diferenciem em células de sustentação, que são as células precursoras das células de Sertoli, e se organizem para envolver e confinar as células germinativas, originando assim os cordões testiculares (Heyn *et al.*, 1998). Em ratos, entre o dia gestacional (DG) 13 e 14, as células de sustentação fetais começam a produzir o hormônio anti-mulleriano (AMH) (Hirobe *et al.*, 1992; Haider, 2004), processo que ocorre sincronicamente com a formação dos cordões testiculares e que tem como função inibir o desenvolvimento dos ductos paramesonéfricos (Josso *et al.*, 1979; Griswold e Behringer, 2009).

As células de Leydig, originadas a partir de células mesenquimais, começam a sintetizar e secretar andrógenos, principalmente a testosterona (Dyche, 1979; Habert e Picon, 1984; Haider, 2004), que estão relacionados com os processos de masculinização e diferenciação dos ductos mesonéfricos no epidídimo, ductos deferentes e glândulas seminais (Huhtaniemi, 1994; Edwards *et al.*, 2006; Griswold e Behringer, 2009). A partir deste momento, a testosterona é convertida a sua forma mais potente, a diidrotestosterona, pela ação da enzima 5α-redutase que direciona a diferenciação do seio urogenital na próstata, diferenciação do tubérculo genital em falo primordial, e posteriormente na glande peniana, além de induzir a formação do escroto (Wilson e Siiteri, 1973).

Considerando que não há a síntese de andrógenos nas fêmeas durante o período gestacional e que as células somáticas não apresentam o gene SRY e cromossomo Y (apresentam um par de cromossomos sexuais XX) (Welsh *et al.*, 2008; Griswold e Behringer, 2009), ocorre a degeneração dos ductos mesonéfricos no dia DG 17, em ratos (George e Wilson, 1994; Huhtaniemi, 1994; Drews, 2000). Nas fêmeas, as células somáticas da gônada primitiva se diferenciam em células foliculares que circundam as células germinativas, originando cistos de células germinativas, e posteriormente, os primeiros folículos ovarianos (Sarraj e Drummond, 2012).

Por sua vez, as células tecais têm origem a partir de células precursoras de fibroblastos do estroma ovariano e são recrutadas por fatores secretados por folículos primários (Young e McNeilly, 2010). Não havendo a síntese do hormônio anti-mulleriano, os ductos paramesonéfricos persistem e vão originar as tubas uterinas, útero, cérvix e a parte anterior da vagina (Griswold e Behringer, 2009; Sarraj e Drummond, 2012).

## 1.7. Desenvolvimento pós-natal testicular

Após o nascimento, os testículos dos mamíferos são formados por cordões sexuais onde se encontram os gonócitos e as células de sustentação, que são os precursores das células germinativas e das células de Sertoli, respectivamente (Setchell e Soder, 2003). As células de Sertoli desempenham um papel importante no suporte e desenvolvimento das células germinativas no interior dos túbulos seminíferos, e atuam na formação da barreira hematotesticular através de junções oclusivas entre células de Sertoli adjacentes (Setchell e Soder, 2003).

Entre os cordões sexuais está localizado o tecido intersticial, onde se encontram as células de Leydig, que são a fonte primordial de andrógenos durante o período perinatal e em toda a fase adulta, e os vasos sanguíneos que têm importante papel no aporte nutricional para as células da linhagem germinativa e somática no testículo (Setchell e Soder, 2003).

Para o desenvolvimento normal da espermatogênese na maturidade sexual é importante que durante o período perinatal os testículos desçam da parede abdominal para o escroto, onde a temperatura é menor em relação à temperatura corporal (Setchell e Soder, 2003). Na espécie humana, o testículo já está completamente inserido no interior do escroto ao nascimento, porém em algumas espécies de mamíferos, tal como ocorre no rato, isso não está completamente estabelecido ao nascimento e a descida testicular nestes animais ocorre mais tarde, entre o nascimento e a instalação da puberdade (Setchell e Soder, 2003).

As células germinativas presentes nos túbulos seminíferos constituem a maior parte do testículo, correspondendo a cerca de 70% do testículo de homens adultos e uma proporção muito menor do que 50% em crianças menores que um ano de idade (Figura 6) (Setchell e Soder, 2003). A contribuição das células germinativas para o volume testicular começa a aumentar com o início da puberdade, a partir do momento em que os túbulos seminíferos começam a obter luz e a espermatogênese se inicia (Setchell e Soder, 2003).

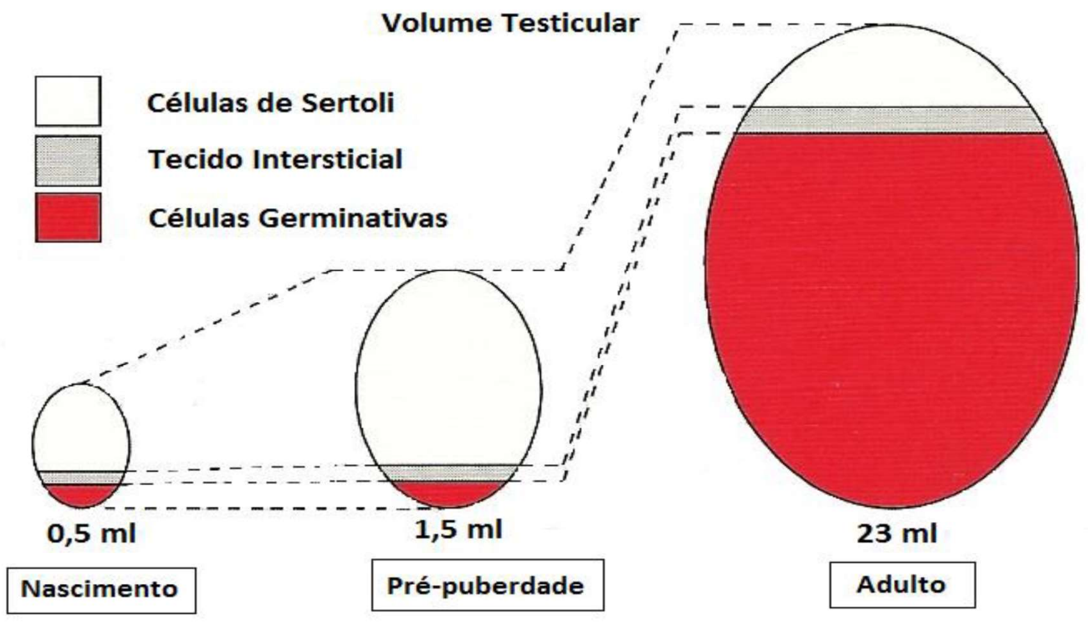


**Figure 6.** Contribuição das células de Sertoli, do tecido intersticial e das células germinativas para o volume testicular em diferentes momentos da vida pós-natal do homem. Imagem modificada. (Setchell e Soder, 2003).

Os túbulos seminíferos são circundados por uma camada de tecido peritubular formada de uma camada acelular (membrana basal) rica em fibras reticulares e uma camada celular composta de células mióides que apresentam capacidade contrátil devido a presença de alfa-isoactina e miosina de músculo liso (Maekawa *et al.*, 1996; Losinno *et al.*, 2012). As células mióides desempenham papel importante para o transporte dos espermatozoides e do fluido testicular ao longo dos túbulos seminíferos (Maekawa *et al.*, 1996; Losinno *et al.*, 2012).

No interior dos túbulos seminíferos estão alocadas as células germinativas e as células de Sertoli, e dentre as células germinativas, encontram-se em contato com a membrana basal as várias linhagens de espermatogônias, tais como: espermatogônias A escuras ou espermatogônias tronco, espermatogônias A pálidas ou espermatogônias intermediárias e espermatogônias B (Rey, 2003; França *et al.*, 2005). As espermatogônias tronco se dividem para manter o número de espermatogônias e fornecer populações celulares para serem destinadas à formação dos gametas (Rey, 2003; França *et al.*, 2005).

Acima das espermatogônias são encontrados as células meióticas, tais como os espermatócitos primários e secundários, e mais voltado para a luz do túbulo seminífero são encontradas as espermátides haploides que terminaram a meiose e começam o processo de espermiogênese, passando por várias alterações morfológicas que vão desde o alongamento dessas espermátides e o aparecimento do acrossomo, até a compactação da cromatina (Rey, 2003; França *et al.*, 2005). Finalmente, as espermátides quando maduras podem ser liberadas do epitélio seminífero e conduzidas até os ductos eferentes e posteriormente ao epidídimo, para passarem pelo processo de maturação (Setchell e Soder, 2003).

Antes da puberdade, os túbulos seminíferos apresentam somente células de Sertoli e as espermatogônias, sendo que estas últimas têm origem a partir dos gonócitos (Rey, 2003). Na peripuberdade começam a aparecer os primeiros espermatócitos no interior dos túbulos seminíferos, e nos seres humanos, os primeiros espermatozoides só são encontrados na urina em torno dos 14 anos, o que indica que a espermatogênese se inicia com a instalação da puberdade (Marty *et al.*, 2003; Setchell e Soder, 2003).

Durante o período fetal as células de Sertoli circundam as células germinativas e com o início da espermatogênese, as espermatogônias passam a se dispor entre as células de Sertoli (Setchell e Soder, 2003). A partir do momento em que as espermatogônias se dividem e começam a formar os primeiros espermatócitos, é importante que estas células germinativas recémformadas consigam passar pelas junções oclusivas das células de Sertoli e saiam do compartimento basal para atingir o compartimento adluminal para dar sequência ao processo espermatogênico (Cheng e Mruk, 2012).

A barreira hematotesticular é formada pelas junções oclusivas entre células de Sertoli adjacentes e tem como função restringir a entrada de substâncias advindas do sangue e de componentes do fluido intersticial para dentro do túbulo seminífero, permitindo a manutenção das condições ideais para que ocorra a meiose e protegendo as células haplóides do sistema imune, porque que neste momento estas células já são consideradas como corpos estranhos pelo sistema imunológico (Setchell, 1980; Setchell e Soder, 2003; Cheng e Mruk, 2012).

Além do papel importante na manutenção da espermatogênese, as células de Sertoli têm papel fundamental na capacidade da produção espermática testicular, porque cada célula de Sertoli consegue sustentar um número máximo de células germinativas, e deste modo, a partir do número total de células de Sertoli é possível estimar a produção espermática de cada testículo (Setchell e Soder, 2003). Nos ratos, estas células se dividem somente até o dia pós-natal (DPN) 15 e nos humanos o número máximo de células de Sertoli parece não ser mais alterado a partir do nascimento (Setchell e Soder, 2003).

Nos roedores, o tecido intersticial é formado por grandes sinusóides linfáticos localizados ente os túbulos seminíferos, os quais circundam as células de Leydig, os macrófagos e os capilares adjacentes (Fawcett *et al.*, 1973; Hutson, 1990; Setchell e Soder, 2003). Nos seres humanos, o tecido intersticial é composto principalmente de tecido conjuntivo frouxo, onde se encontram vasos linfáticos pequenos e discretos, as células de Leydig, os macrófagos e os mastócitos (Fawcett *et al.*, 1973; Setchell e Soder, 2003).

Nos humanos e nos ratos, o número máximo de células de Leydig é atingido ainda durante a gestação, devido a importância da secreção de andrógenos para a diferenciação do sistema genital masculino, sendo que após o nascimento o número de células de Leydig torna-se parcialmente menor (Setchell e Soder, 2003).

Nos humanos têm sido descritos quatro tipos de células de Leydig: as fetais, as infantis, as puberais e as adultas (Nistal *et al.*, 1986). As células de Leydig fetais secretam andrógenos e estão presentes no testículo até por volta dos 3-6 meses de vida pós-natal (Nistal *et al.*, 1986). Estas células gradativamente se diferenciam e são substituídas pelas células de Leydig infantis, que não secretam andrógenos e que são encontradas no interstício até por volta dos 8 anos de idade (Nistal *et al.*, 1986). Ao entrar na puberdade, as células de Leydig infantis da criança se diferenciam em células de Leydig puberais e posteriormente, em células de Leydig adultas, as quais apresentam secreção de andrógenos em resposta ao aumento do LH plasmático que ocorre após a instalação da puberdade (Nistal *et al.*, 1986).

Nos ratos ocorre processo semelhante e as células de Leydig fetais são substituídas pelas células de Leydig progenitoras, posteriormente pelas células de Leydig imaturas e finalmente pelas células de Leydig adultas (Chen *et al.*, 2009).

### 1.8. Morfofisiologia, tipos celulares e desenvolvimento pós-natal epididimário

Nos mamíferos adultos, o epidídimo é um ducto único altamente enovelado que é formado por epitélio pseudoestratificado cilíndrico com estereocílios e apresenta várias funções, tais como, a secreção de substâncias que vão atuar na maturação espermática; a reabsorção de fluido, proteínas e outros compostos orgânicos; a proteção do espermatozoide que está percorrendo todo o epidídimo contra as células do sistema imune; e a concentração e armazenamento dos espermatozoides (Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012).

A aquisição destas funções pelo epidídimo é acompanhada de uma série de alterações morfológicas durante o seu desenvolvimento pós-natal, resultando em um ducto único que apresenta várias regiões com diferenças morfológicas, funcionais e de expressão gênica ao longo do órgão (Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012).

Dependendo da espécie de mamífero, o epidídimo pode ser subdividido nas seguintes regiões: segmento inicial, zona intermediária, cabeça, corpo e cauda, sendo que cada região apresenta características morfológicas específicas, podendo inclusive a constituição celular ser diferente ao longo do ducto epididimário, dependendo da região em questão (Hermo e Robaire, 2002). As células que formam o epitélio epididimário são classificadas como: células principais, estreitas, apicais, claras, basais e halo, de acordo com a localização, as características celulares e a abundância dessas células no epitélio (Hermo e Robaire, 2002).

As células principais constituem a maior população celular no epidídimo e podem representar de 65-80% da população celular total e diferir ultraestrutural e funcionalmente ao longo das várias regiões do órgão, de modo que essas diferenças estão relacionadas à aparência e organização das organelas associadas com a secreção celular e a endocitose (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012). Atualmente tem sido relatado que as células principais não se dividem na vida adulta e que elas são responsáveis pela secreção de todas as proteínas liberadas para a luz do ducto epididimário, tendo papel importante na maturação espermática (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012).

As células estreitas estão presentes no epidídimo do rato adulto somente no segmento inicial e na zona intermediária, são mais estreitas que as células principais e apresentam função relacionada com a endocitose e a secreção de $H^+$ para a luz do órgão (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012).

Por sua vez, as células apicais são encontradas primordialmente no segmento inicial e na zona intermediária do epidídimo do rato, embora possam ser encontradas em outras regiões epididimárias em ratos com idade avançada (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012). Estas células são caracterizadas morfologicamente pela sua localização mais apical no epitélio, ou seja, não apresentam contato com a membrana basal, diferentemente do que ocorre com as células principais, basais, claras e estreitas (Hermo e Robaire, 2002). A literatura é escassa sobre a função destas células, no entanto é descrito que elas realizam endocitose de substâncias da luz epididimária (Hermo e Robaire, 2002).

As células claras estão presentes na cabeça, corpo e cauda epididimária e apresentam-se com o citoplasma opaco em preparações histológicas usuais como a Hematoxilina-eosina (HE), sem demonstrar afinidade por corantes ácidos ou básicos (Arrotéia *et al.*, 2012). Este tipo celular apresenta ampla atividade endocítica, obtendo várias proteínas a partir da luz do epidídimo e fagocitando as gotas citoplasmáticas liberadas pelos espermatozoides ao longo do trânsito epididimário, as quais são trazidas desde a liberação dos espermatozoides do epitélio seminífero (Hermo e Robaire, 2002).

Em relação às células basais, sabe-se que elas têm contato direto com a membrana basal, que não possuem contato direto com o lúmen do ducto e que estão presentes ao longo de todo o epidídimo e também no ducto deferente (Hermo e Robaire, 2002). As células basais secretam várias substâncias que acredita-se que possa regular a função das células principais (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012).

Por fim, as células halo estão presentes ao longo de todo o epidídimo do rato e estão localizadas normalmente na base do epitélio, apresentando morfologia característica devido ao halo citoplasmático claro presente no seu citoplasma (Hermo e Robaire, 2002). As células halo têm sido descritas como monócitos ou linfócitos (linfócitos T auxiliares ou linfócitos T citotóxicos) que se alocam no epidídimo e que com o avanço da idade podem aumentar em número e ser encontradas como linfócitos B e neutrófilos, o que não é comum em animais mais jovens (Hermo e Robaire, 2002; Arrotéia *et al.*, 2012). A presença destas células sugere que elas podem exercer uma função imunológica no epitélio epididimário (Hermo e Robaire, 2002).

Desta forma, os diferentes tipos celulares do epitélio epididimário encontram-se ilustrados nas figuras abaixo:

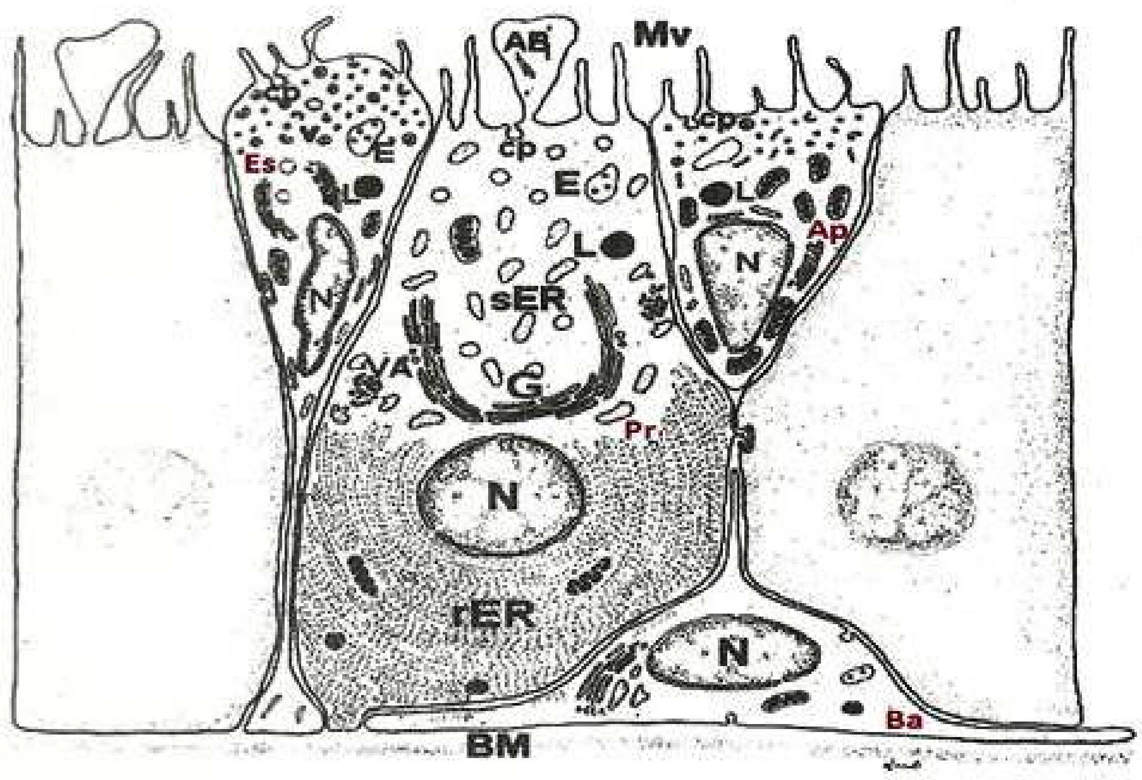


**Figura 7.** Distribuição celular no segmento inicial do epidídimo. Pr: célula principal; Ba: célula basal; Ap: célula apical; Es: célula estreita. Imagem modificada (Hermo e Robaire, 2002).

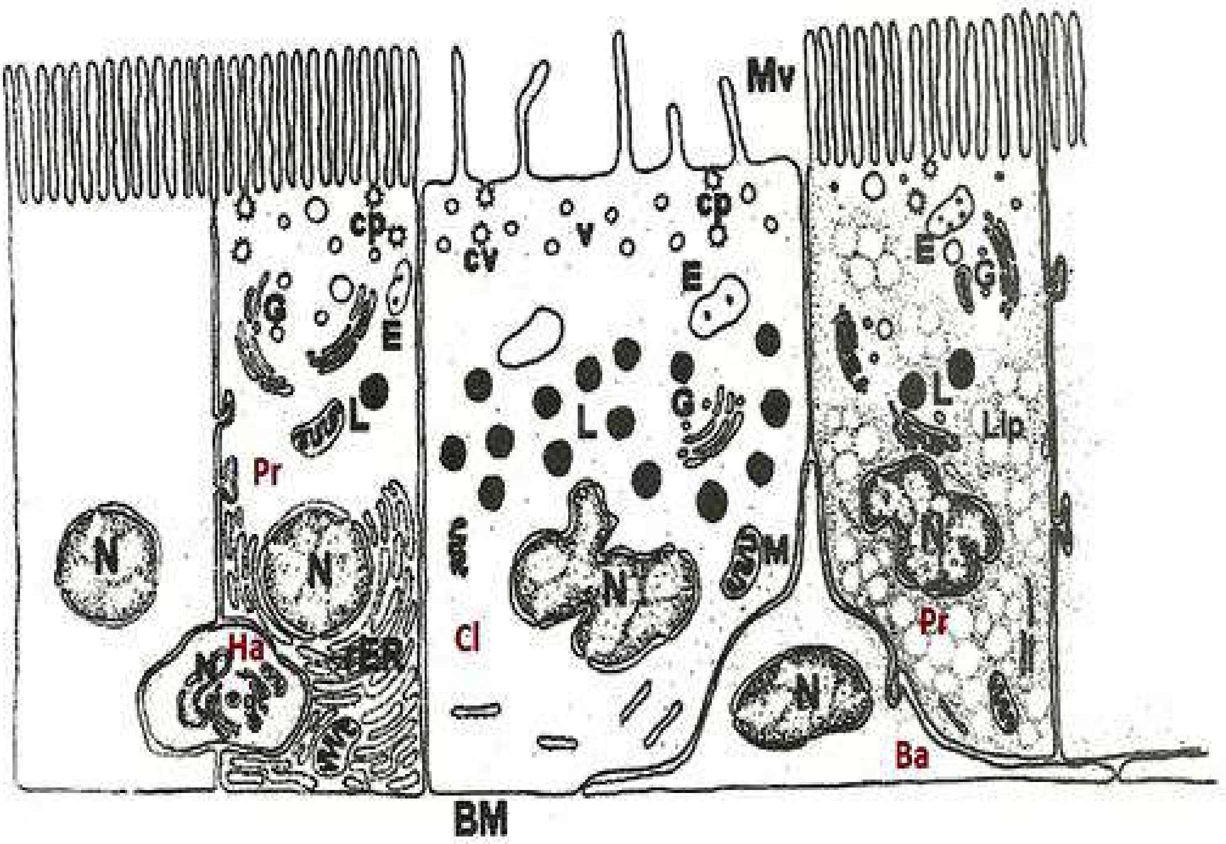


**Figure 8.** Distribuição celular na região da cabeça/corpo do epidídimo. Ha: célula halo; Cl: célula clara; Ba: célula basal; Pr: célula principal. Pr da região da cabeça do epidídimo à esquerda (apresenta grande quantidade de retículo endoplasmático rugoso) e Pr da região do corpo à direita (presença de muitas gotículas lipídicas). Imagem modificada (Hermo e Robaire, 2002).

O epidídimo do rato é um dos mais estudados e é utilizado como modelo para estudo da diferenciação epididimária, e além disso, os processos de diferenciação parecem ser bem semelhantes entre as espécies de mamíferos (Rodríguez *et al.*, 2002). O desenvolvimento pós-natal do epidídimo é dividido em três períodos, que compreendem o período indiferenciado, o período de diferenciação e o período de expansão (Rodríguez *et al.*, 2002). As alterações ao longo do desenvolvimento do epidídimo consistem na diferenciação e aquisição dos vários tipos celulares e na compartimentalização das diferentes regiões epididimárias (Hermo e Robaire, 2002). As alterações morfológicas que ocorrem durante o desenvolvimento pós-natal do epidídimo do rato encontram-se ilustradas na figura abaixo.

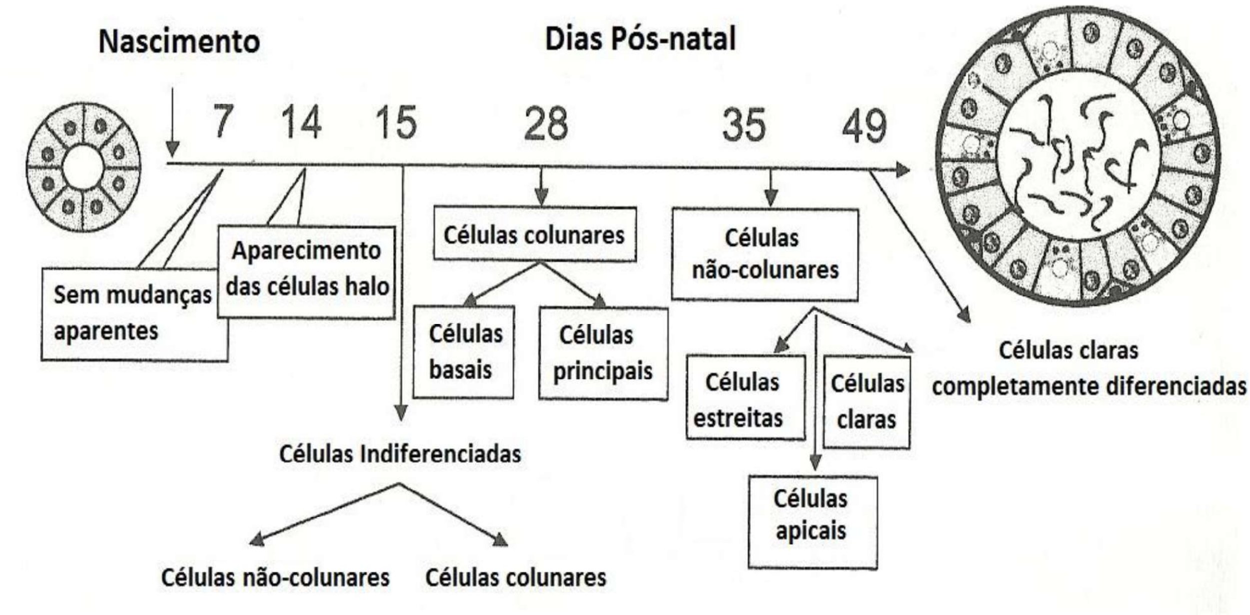


**Figura 9.** Desenvolvimento pós-natal do epidídimo do rato. Imagem modificada (Rodríguez *et al.*, 2002).

No período indiferenciado do epidídimo do rato que compreende o período do DPN 1 ao DPN 15, as células que compõem o epitélio epididimário ainda são indiferenciadas e são caracterizadas histologicamente como células colunares baixas que não apresentam estereocílios (Hermo *et al.*, 1992; Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012). O epitélio apresenta abundância de figuras mitóticas e as células apresentam muitos ribossomos livres e polirribossomos, porém poucas mitocôndrias estão presentes nesse período a nível ultraestrutural (Rodríguez *et al.*, 2002). Além disso, cerca de metade das junções oclusivas do epitélio (40-60%) já estão formadas nessa fase e as células halo começam a aparecer no dia DPN 14 (Rodríguez *et al.*, 2002).

No período de diferenciação do epidídimo, que no rato vai do DPN 16 ao DPN 44, o epitélio epididimário passa por muitas modificações, de modo que o epitélio indiferenciado começa a se diferenciar e surgem as primeiras células colunares e as primeiras células não-colunares no epididímo no DPN 21 (Hermo *et al.*, 1992; Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012). Em seguida, as células colunares se diferenciam em células basais e principais no DPN 28 e as células principais começam a aumentar em tamanho e adquirir aspecto de célula colunar alta no DPN 35 (Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012). As células principais neste momento já exibem aparelho de Golgi bem elaborado, borda em escova, muitas vesículas em seu citoplasma e demonstram endocitose ativa (Rodríguez *et al.*, 2002).

Ao final do período de diferenciação, as células não-colunares originarão as células estreitas, apicais e as células claras (Arrotéia *et al.*, 2012), sendo que no DPN 36 ocorre o desaparecimento das células estreitas da cabeça, corpo e cauda epididimária, ficando restritas somente ao segmento inicial (Rodríguez *et al.*, 2002).

A proliferação celular no epitélio atinge seu pico no período de diferenciação entre o DPN 21 e DPN 28 no segmento inicial e entre o DPN 14 e DPN 28 na cabeça, corpo e cauda, sendo que o fluido testicular que penetra o epidídimo tem papel importante na estimulação da atividade proliferativa, principalmente no segmento inicial (Hermo *et al.*, 1992; Rodríguez *et al.*, 2002).

No período de expansão, que se inicia no DPN 44 e vai até a fase adulta, as mudanças mais evidentes envolvem a presença de espermatozoides no lúmen do ducto epididimário e o aumento no tamanho do epidídimo, devido ao aumento no comprimento e no peso do órgão (Rodríguez *et al.*, 2002). As células claras originadas no final do período de diferenciação já se apresentam diferenciadas ao final do DPN 49 e o epitélio epididimário mostra-se totalmente diferenciado neste momento (Hermo *et al.*, 1992; Rodríguez *et al.*, 2002).

O desenvolvimento epididimário requer níveis aumentados de andrógenos provenientes das células de Leydig e a diferenciação do epitélio depende também de fatores provenientes do fluido testicular e/ou da região proximal do epidídimo (Hermo *et al.*, 1992; Rodríguez *et al.*, 2002; Marty *et al.*, 2003). As mudanças que ocorrem ao longo do desenvolvimento do epidídimo necessitam da expressão de genes chave e a síntese de proteínas codificadas por esses genes para a formação de um ambiente luminal favorável e capaz de permitir a maturação e o armazenamento dos espermatozoides (Rodríguez *et al.*, 2002).

A expressão dos receptores de andrógenos aumenta no epitélio epididimário de acordo com o desenvolvimento pós-natal do órgão, demonstrando assim a importância da expressão desses receptores nas células epididimárias para o desenvolvimento normal do epidídimo (You e Sar, 1998; Perobelli *et al.*, 2013). Além disso, os receptores de estrógenos desempenham um papel importante na diferenciação dos ductos eferentes e na reabsorção de fluido testicular, o que está diretamente relacionado com a maturação e concentração espermática (Rodríguez *et al.*, 2002; Arrotéia *et al.*, 2012).

Durante o desenvolvimento epididimário ocorre a formação da barreira hemato-epididimária através das junções oclusivas entre as células principais adjacentes, a qual deve estar totalmente formada antes da chegada dos primeiros espermatozoides na luz do ducto (Rodríguez *et al.*, 2002). As junções oclusivas desempenham papel importante para a fisiologia normal do epitélio epididimário, sendo que no rato a barreira hemato-epididimária já está estruturalmente pronta no DPN 21 (Rodríguez *et al.*, 2002).

### 1.9. Instalação da puberdade nas fêmeas e desenvolvimento pós-natal ovariano

A instalação da puberdade em fêmeas corresponde ao início de eventos que direcionam a anatomia e a fisiologia do sistema reprodutor feminino (Andersson *et al.*, 2013). Para que ocorra a instalação da puberdade nas fêmeas é necessário que os níveis de FSH secretados pela hipófise aumentem, levando ao início da maturação ovariana, que é acompanhada de níveis aumentados de estrógenos (Shaikh, 1971; Andersson *et al.*, 2013; Guerra *et al.*, 2017). Desta forma, a abertura vaginal e o primeiro estro têm sido usados como importantes sinais externos da instalação da puberdade e têm sido amplamente correlacionados com os níveis de estradiol (U.S. Environmental Protection Agency, 1996).

Com o aumento nos níveis de estrógenos ocorre uma retroalimentação positiva estimulando a secreção e a formação de um pico de LH, e assim, ocorre a ovocitação e a luteinização das células foliculares da camada granulosa e das células que formam a teca interna (Andersson *et al.*, 2013). A luteinização é o processo responsável pela formação do corpo lúteo, que tem como função principal sintetizar e secretar progesterona na segunda metade do ciclo sexual (Andersson *et al.*, 2013).

A maturação ovariana de ratas é resultado de uma cascata de eventos neuroendócrinos que incluem o estabelecimento de níveis pulsáteis de LH e aumento nas concentrações de prolactina e GH, que se iniciam por volta do DPN 30 e culminam na instalação da puberdade nas fêmeas (Andrews e Ojeda, 1981; Westwood, 2008). Em ratas recém-nascidas, o ovário é formado somente por folículos primordiais e primários, já não havendo mais ovogônias no tecido ovariano (Davis *et al.*, 2001; Zhang *et al.*, 2016). Por volta do DPN 20, já há uma abundância de folículos pré-antrais, folículos que apresentam várias camadas de células foliculares e ausência de antro folicular, e presença de poucos folículos antrais, caracterizados pela presença de antro folicular (Picut et al. 2014a; Picut et al. 2014b). Entre o DPN 27 e o DPN 29 começam a aparecer os primeiros folículos de Graaf, também denominados folículos maduros, e aumenta-se a necrose de ovócitos e a atresia folicular próximo a região medular do órgão (Picut et al. 2014a; Picut et al. 2014b). O número de corpos lúteos aumenta significativamente em ratas pré-puberes com o avanço no desenvolvimento puberal, de modo que o número dessas estruturas endócrinas está diretamente relacionado com o grau de desenvolvimento e maturação ovariana (Picut et al. 2014a; Picut et al. 2014b).

O ovário de fêmeas adultas é dividido em duas regiões, sendo elas a região cortical, aonde se encontram os folículos ovarianos, e a região medular, que é formada por tecido conjuntivo e vasos sanguíneos (Bukovsky *et al.*, 2005; Picut *et al.*, 2014b). No ovário de rata adulta são encontrados todos os estágios foliculares, assim como corpos lúteos, em todas as fases do ciclo estral (Westwood, 2008). Entretanto, os corpos lúteos mostram diferenças na sua morfologia dependendo da fase do ciclo estral, podendo se apresentar mais basófilos e apresentando fluido no seu interior, se recém-formados, ou então serem mais acidófilos e exibirem vacuolização e tecido fibroso no seu interior, quando mais velhos (Westwood, 2008).

### 1.10. Desenvolvimento pós-natal uterino

No primeiro dia de vida pós-natal de ratas, o útero é formado somente por um epitélio luminal cúbico ou colunar baixo, sendo que a diferenciação da musculatura lisa, do estroma endometrial e do epitélio glandular ocorrem durante a vida pós-natal (Sheehan *et al.*, 1981; Branham e Sheehan, 1995). Em torno de uma semana após o nascimento, as glândulas uterinas surgem da invaginação do epitélio uterino e crescem em direção ao estroma endometrial, sendo que este último tem origem a partir do mesênquima uterino (Branham e Sheehan, 1995; Hu *et al.*, 2004). Além disso, também no período pós-natal o mesênquima uterino se diferencia nas duas camadas do miométrio, o qual é formado por uma camada de musculatura lisa longitudinal externa e uma camada circular interna (Brody e Cunha, 1989; Hu *et al.*, 2004).

Entre o DPN 20 e DPN 26, o útero mostra-se quiescente e com ausência de leucócitos no estroma endometrial e ausência de degeneração do epitélio luminal e glandular uterino (Picut *et al.*, 2014a). No período entre o DPN 27 e DPN 38, o epitélio luminal uterino começa a se alongar e inicia-se uma degeneração natural em células epiteliais, porém os leucócitos ainda não estão presentes no estroma endometrial da rata (Picut *et al.*, 2014a). No período que corresponde entre o DPN 39 e DPN 43, o epitélio uterino torna-se mais alto (colunar), a degeneração em células epiteliais torna-se mais comum e os primeiros leucócitos começam a se infiltrar e aparecer no estroma endometrial uterino (Picut *et al.*, 2014a).

Quando já se encontra estabelecida a ciclicidade estral na rata, o útero começa a mostrar diferentes aspectos morfológicos que variam de acordo com a fase do ciclo estral (Westwood, 2008). Na fase de proestro, o epitélio luminal uterino varia de cúbico a colunar, as mitoses são frequentes nas células epiteliais e há pouca degeneração dessas células, além de pouco infiltrado de células inflamatórias (Westwood, 2008). Durante a fase de estro é comum a ocorrência de necrose de células do epitélio luminal uterino e do epitélio glandular, perda da atividade mitótica das células epiteliais, bem como a presença de infiltrados inflamatórios no estroma endometrial (Westwood, 2008). A fase de metaestro é caracterizada pela presença de degeneração e atividade mitótica de células no epitélio luminal uterino, sendo portanto observadas juntas no epitélio (Westwood, 2008). Na última fase, o diestro, observa-se um lúmen reduzido circundado por um epitélio luminal uterino baixo e presença de poucas mitoses no epitélio luminal (Westwood, 2008).

# Justificativa

## JUSTIFICATIVA DO TRABALHO

A puberdade é um período crítico e susceptível à ação de agentes químicos, principalmente em relação aos que podem agir como desreguladores endócrinos, interferindo com o desenvolvimento pós-natal e maturação dos órgãos reprodutores. As estatinas têm sido recomendadas para utilização em crianças e adolescentes que apresentam níveis séricos elevados de colesterol ou risco aumentado de doença cardiovascular, pois promovem a diminuição dos níveis de colesterol total pela inibição da enzima HMG-CoA redutase, além de promover efeitos pleiotrópicos nos indivíduos que fazem uso do medicamento.

Sabendo-se que estudos prévios associam o uso das estatinas com a diminuição nas concentrações séricas de testosterona e com a capacidade de promover várias alterações durante o desenvolvimento reprodutivo, a hipótese do trabalho propõe que a exposição de ratos juvenis à rosuvastatina possa interferir com os parâmetros reprodutivos e a fertilidade das gerações $F_0$ e $F_1$. Havendo indícios de que esses danos possam ser mediados por estresse oxidativo e que as estatinas possam promover efeitos genéticos e epigenéticos, o estudo também apresenta a hipótese de que a suplementação com ácido ascórbico possa reverter, pelo menos parcialmente, os danos reprodutivos promovidos pela rosuvastatina nas gerações $F_0$ e $F_1$.

# *Objetivos*

*Objetivo Geral*

Investigar os parâmetros reprodutivos e a fertilidade das gerações $F_0$ e $F_1$ de ratos machos pré-púberes expostos ao agente hipolipemiante rosuvastatina e avaliar o papel do ácido ascórbico na prevenção desses efeitos adversos.

*Objetivos Específicos*

✓ Expor ratos pré-puberes a diferentes doses de rosuvastina na ausência ou presença de ácido ascórbico e investigar os parâmetros imediatos e tardios sobre o sistema genital e a fertilidade das gerações $F_0$ e $F_1$ (Geração $F_1$: prole masculina e feminina).

✓ Avaliar nos machos da geração $F_0$ o período de instalação da puberdade, o comportamento sexual e o teste de fertilidade por acasalamento natural; dosagens de hormônios sexuais; análises espermáticas (motilidade, morfologia e contagens espermáticas); histologia de testículo e epidídimo; imunohistoquímica para AR e ERS1, e ensaio TUNEL no testículo; ensaio cometa para os espermatozoides; avaliação ultraestrutural dos espermatozoides e atividade de enzimas antioxidantes no testículo e epidídimo.

✓ Investigar nos machos da geração $F_1$ os seguintes parâmetros: comportamento sexual, fertilidade por acasalamento natural, dosagens de hormônios sexuais, análises espermáticas, ensaio TUNEL no testículo, histologia testicular e epididimária, e ensaio cometa para os espermatozoides.

✓ Avaliar nas fêmeas da geração $F_1$ o tempo de instalação da puberdade, peso de órgãos reprodutores e vitais, regularidade do ciclo estral, quantificação folicular ovariana, morfometria uterina, análise histopatológica de ovários e útero, dosagens hormonais, análise do comportamento sexual e teste de fertilidade.

# Capítulo 1

**Manuscrito I**

O primeiro manuscrito é intitulado **“Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty”** de autoria de Gabriel Adan Araújo Leite, Thamiris Moreira Figueiredo, Marciana Sanabria, Ana Flávia Mota Gonçalves Dias, Patrícia Villela e Silva, Airton da Cunha Martins Junior, Fernando Barbosa Junior e Wilma De Grava Kempinas, publicado na revista Reproductive Toxicology, volume 73, 10ª edição, páginas 328-338, 2017. Elsevier, ISSN: 0890-6238. Fator de impacto: 2,341.

Reproductive Toxicology 73 (2017) 328–338

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty

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A R T I C L E   I N F O

*Article history:*
Received 30 November 2016
Received in revised form 23 May 2017
Accepted 12 July 2017
Available online 18 July 2017

*Keywords:*
Rosuvastatin
Ascorbic acid
Puberty
Male reproduction
Testis and epididymis

A B S T R A C T

Dyslipidemias are occurring earlier in the population due to the increase of obesity and bad eating habits. Rosuvastatin inhibits the enzyme HMG-CoA reductase, decreasing total cholesterol. Ascorbic acid is an important antioxidant compound for male reproductive system. This study aimed to evaluate whether ascorbic acid supplementation may prevent the reproductive damage provoked by rosuvastatin administration at prepuberty. Male pups were distributed into six experimental groups that received saline solution 0.9%, 3 or 10 mg/kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 150 mg/day of ascorbic acid associated with 3 or 10 mg/kg/day of rosuvastatin from post-natal day (PND) 23 until PND53. Rosuvastatin-treated groups showed delayed puberty installation, androgen depletion and impairment on testicular and epididymal morphology. Ascorbic acid partially prevented these reproductive damages. In conclusion, rosuvastatin exposure is a probable risk to reproductive development and ascorbic acid supplementation may be useful to prevent the reproductive impairment of rosuvastatin exposure.



## 1. Introduction

Dyslipidemias have been found earlier in the population due to the increase of obesity and have been common in the children and adolescents that present slight or moderate obesity and consequently increased levels of serum LDL-cholesterol, VLDL-cholesterol and diminished levels of HDL-cholesterol [1], as well as an augmentation of serum triglycerides [2].

The main causes of the triggering of infant dyslipidemias are related to inappropriate life style such as sedentary, bad eating habits and other factors associated with children life style [3].

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutharyl coenzyme A reductase (HMG-CoA reductase), which is a crucial enzyme to the cholesterol biosynthesis [1,4], thus statins are responsible to decrease total cholesterol and LDL-cholesterol [4–6]. Since statins impede the conversion of HMG-CoA to mevalonate, they diminish endogenous cholesterol formation [7,8]. These drugs also inhibit the synthesis of important compounds, named intermediate isoprenoids, such as the geranyl-geranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) [7,8].

Furthermore, HMG-CoA reductase inhibitors have been shown pleiotropic effects, thus they have been recommended due to their anti-inflammatory and immune modulators effects, blood protector effects for the treatment of brain diseases, such as depression [9,10], besides their proliferation inhibition and induction of cancer cell death capability [11–14].

Rosuvastatin shows various pharmacological advantages in comparison with the other statins and provides unique binding characteristics with the enzyme HMG-CoA reductase, leading to superior cholesterol inhibitory effects in relation to the other statins [15,16].

Puberty is a critical period in which is occurring fast neuroendocrine alterations and important morphological changes in

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http://dx.doi.org/10.1016/j.reprotox.2017.07.006
0890-6238/

**Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty**

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Abstract

Dyslipidemias are occurring earlier in the population due to the increase of obesity and bad eating habits. Rosuvastatin inhibits the enzyme HMG-CoA reductase, decreasing total cholesterol. Ascorbic acid is an important antioxidant compound for male reproductive system. This study aimed to evaluate whether ascorbic acid supplementation may prevent the reproductive damage provoked by rosuvastatin administration at prepuberty. Male pups were distributed into six experimental groups that received saline solution 0.9%, 3 or 10 mg/Kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin from post-natal day (PND) 23 until PND53. Rosuvastatin-treated groups showed delayed puberty installation, androgen depletion and impairment on testicular and epididymal morphology. Ascorbic acid partially prevented these reproductive damages. In conclusion, rosuvastatin exposure is a probable risk to reproductive development and ascorbic acid supplementation may be useful to prevent the reproductive impairment of rosuvastatin exposure.

Keywords: Rosuvastatin, ascorbic acid, puberty, male reproduction, testis and epididymis.

1. Introduction

Dyslipidemias have been found earlier in the population due to the increase of obesity and have been common in the children and adolescents that present slight or moderate obesity and consequently increased levels of serum LDL-cholesterol, VLDL-cholesterol and diminished levels of HDL-cholesterol [1], as well as an augmentation of serum triglycerides [2].

The main causes of the triggering of infant dyslipidemias are related to inappropriate life style such as sedentary, bad eating habits and other factors associated with children life style [3].

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutharyl coenzyme A reductase (HMG-CoA reductase), which is a crucial enzyme to the cholesterol biosynthesis [1,4], thus statins are responsible to decrease total cholesterol and LDL-cholesterol [4–6]. Since statins impede the conversion of HMG-CoA to mevalonate, they diminish endogenous cholesterol formation [7,8]. These drugs also inhibit the synthesis of important compounds, named intermediate isoprenoids, such as the geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) [7,8].

Furthermore, HMG-CoA reductase inhibitors have been shown pleiotropic effects, thus they have been recommended due to their anti-inflammatory and immune modulators effects, blood protector effects for the treatment of brain diseases, such as depression [9,10], besides their proliferation inhibition and induction of cancer cell death capability [11–14].

Rosuvastatin shows various pharmacological advantages in comparison with the other statins and provides unique binding characteristics with the enzyme HMG-CoA reductase, leading to superior cholesterol inhibitory effects in relation to the other statins [15,16].

Puberty is a critical period in which is occurring fast neuroendocrine alterations and important morphological changes in mammals, as well as, is considered an important stage for reproductive health [17,18]. During puberty installation spermatogenesis and steroidogenesis are developing and are not established yet, then it makes male reproductive tract more susceptible to the chemical agents named endocrine disruptors [19,20]. These chemical compounds may diminish androgen function and delay male reproductive tract development and the adverse reproductive effects induced by these toxic compounds are dependent to the time of exposure [21].

Children and adolescents are exposed to several chemical agents that may alter puberty timing, development and acquisition of reproductive function [17]. Since puberty timing alterations may affect reproductive capability in the adult life, these alterations provide relevant information about the reproductive function at sexual maturity [22,23].

The utilization of substances with higher potential of antioxidant activity or another mechanism that may prevent toxic effects from chemical agents has been widely investigated [24–27], since many drugs are necessary to treat diseases and show adverse side effects that are not interesting during the treatment [25,27].

Ascorbic acid is a hydrosoluble vitamin that is necessary for many biochemical reactions in the organism and presents several biological functions, such as its role as essential nutrient to collagen biosynthesis and α-tocopherol recycling [28–30]. On male reproductive system ascorbic acid acts like an antioxidant compound against the oxidative stress in the testis and plays a protecting role for spermatogenesis [29,31–33].

Previous study showed that rosuvastatin administration at prepuberty diminished testosterone concentrations, delayed puberty installation, provoked testicular and epididymal morphological alterations and reduction in the expression of androgen receptors in both testis and epididymis [34]. Considering the use of statins by children and adolescents and the delayed reproductive development provoked by rosuvastatin in male rats, as reported before [34], the present study aims to diminish or prevent the reproductive adverse effects produced by rosuvastatin exposure during prepubertal phase, using ascorbic acid supplementation concomitantly.

## 2. Material and Methods

### 2.1. Animals

#### 2.1.1. Obtention of pups and reduction of litters

Male and female (45 days of age) nonpathogenic free Wistar rats were supplied from Central Biotherium of State University of São Paulo (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu.

Rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Animals were maintained under controlled temperature

(23 ± 1°C) and lighting conditions (12:12h photoperiod) and their health was monitored along the experiment. Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During sexual maturity two nulliparous female rats (75 days of age) were mated with one male (90 days of age) during the dark phase of the lighting cycle and the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating female rats were maintained in individual cages.

After birth, the number of pups per litter was culled to eight on post-natal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not considered into the experimental protocol and were euthanized.

2.1.2. Experimental design

On PND 23 male pups were distributed into six experimental groups (n=10 per group, with one or two pups per litter for each group), that received vehicle (control group, saline solution 0.9%), supplementation with 150 mg/day of ascorbic acid (AA), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0.9% (3 mg or 10 mg) or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin (3 mg + AA or 10 mg + AA). Ascorbic acid and rosuvastatin were purchased from Farmácia Botica Oficinal (Botucatu/Brazil). The treatments were administered by gavage since PND 23 until PND 53, following male pubertal assay of 31 days recommended by Environmental Protection Agency (EPA) [17].

The doses of rosuvastatin available to diminish total cholesterol and LDL-cholesterol often used by humans are between 5 and 40 mg/day [35], thus the doses utilized in this study were based on body surface area correction from children available doses of rosuvastatin to juvenile rats equivalent doses [36]. The doses of ascorbic acid supplementation were based on previous studies [29,30]. During the treatment, the rats were monitored in relation to the indications of distress, such as the presence of bristling hair and the ingestion of food and water. Individual pup weights were registered during the exposure period.

The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

Male rats were euthanized on PND 53 after puberty onset confirmation by preputial separation, in order to evaluate the short-term reproductive effects due to rosuvastatin exposure and/or ascorbic acid supplementation. Therefore, the following parameters were obtained: final body weight, reproductive and vital organ weights, testicular and epididymal histopathology, total cholesterol, triglycerides and hormonal serum concentrations and antioxidant activity on testis and epididymis.

2.2. Puberty installation

Preputial separation is a parameter used as a sign of puberty installation and was investigated daily since PND 30, based on manual retraction of prepuce [37]. In this manner, total retraction of penile glans indicated that the animals have completed preputial separation.

2.3. Euthanasia of the rats, body weight and organ weights

Male rats were weighed on PND 53 and euthanized following narcosis by $CO_2$ asphyxiation and thereafter, blood was collected by inferior vena cava, between 9:00 and 11:30 a.m. Reproductive organs, such as, left testis, epididymis and vas deferens, seminal gland (full and empty, without the coagulating gland) and ventral prostate, from all rats were collected and weighed. Vital organs that have an important role for toxicological parameters, such as kidney, adrenal, liver, thyroid, pituitary and brain were also obtained and weighed.

2.4. Serum hormonal concentrations and biochemical analyses

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of biochemical and hormonal determination. Testosterone, FSH and LH were determined by double-antibody radioimmunoassay. LH and FSH used specified kits supplied by National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK) and testosterone concentrations were measured by Testosterone Maia kit (Biochem Immuno System, Allentown, PA). All samples were measured in the same assay to avoid the inter-assay errors. Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone. Total cholesterol and triglycerides were determined using Cobas Mira Plus® Bio200 equipment (Katal® kits).

2.5. Histological procedures

Left testis and epididymis were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 μm (transversal sections of testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE) to evaluate the testis and epididymis morphology under light microscopy. The evaluation was performed in blind assay and the figures were obtained using a light microscopy Leica, coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows.

Seminiferous tubules cross-sections were randomly chosen in three non-serial testicular sections per animal obtained with a distance of 50 μm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium). Interstitial tissue and peritubular myoid cells histopathological analysis were qualitative and the interstitial analysis aimed to assess Leydig cells morphology and the appearance of blood vessels. Epididymal histopathological analysis was also qualitative to evaluate each region of the organ according to the epithelium, lumen and interstitial tissue morphological appearance.

2.6. Maturation degree of seminiferous epithelium

In order to evaluate the maturation degree of the seminiferous epithelium, 100 cross-sections of seminiferous tubules per animal (n = 8 per group), were evaluated randomly, using the adapted method of assigning values according to the type of mature germ cell most numerous in the tubular epithelium: degree 1: spermatocytes I or II; degree 2: young spermatids with rounded nucleus (stage 1 to 8 of spermiogenesis); degree 3: spermatids in maturation phase, with ovoid or elongated nucleus (stage 9 to 14 of spermiogenesis); degree 4: spermatids in maturation phase, with elongated nucleus (stage 15 to 18 of spermiogenesis); degree 5: mature spermatids (stage 19 of spermiogenesis) in small quantity; degree 6: mature spermatids (stage 19 of spermiogenesis) in average amount; degree 7: mature spermatids (stage 19 of spermiogenesis) in larger amount. The number of seminiferous tubules in each degree was multiplied by its degree, and then the values were added and divided by 100, resulting in the “average degree” [27].

2.7. Antioxidant activity

2.7.1 Homogenate protein determination

Right testis and epididymis were homogenized separately in 50 mM Tris-HCl buffer solution (pH-7.4).The protein content in testis and epididymis homogenate was determined by Bradford assay using bovine serum albumin as a standard [38]

2.7.2. Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was assessed using the TBARS assay [39]. Briefly, 200µL of testis or epididymis homogenate were mixed with 500 µL thiobarbituric acid 0,8% , 500 µL acetic acid buffer, 200 µL of SDS 8.1% and 100 µL of water. These samples were then incubated for 2 hours at 95°C and allowed to cool to room temperature. The absorbance was measured at 532nm. The amount of lipid peroxidation was expressed as µM TBARS/mg protein. All of the assays were performed in duplicate.

2.7.3. Reduced glutathione (GSH) levels

Reduced glutathione levels were determined following the procedures reported previously [40]. Briefly, the homogenates were precipitated by adding 100 µl of 30% trichloroacetic acid (TCA) to 500 µl of the homogenate and then centrifuged at 4000×g for 10 min. The reaction mixture contains: 50 µl of the supernatant, 50 µl of 10 mM DTNB and 900 µl of 1 M potassium phosphate buffer, pH 7.4. Absorbance was read at 412 nm and GSH expressed as nmol GSH/mg protein using GSH as standard or a molar extinction coefficient of 13,100 $M^{-1}/cm^{-1}$. The assays were performed in duplicate.

2.7.4 Catalase activity assay

Catalase activity was determined spectrophotometrically based on the rate of decomposition of hydrogen peroxide by catalase [41]. One unit of the enzyme is defined as the amount of enzyme that decomposes 1 mmol hydrogen peroxide in 2 min at 25 °C. Briefly, 900 µl of 50mM phosphate buffer was transferred into a cuvette, and then 35 µl of 0.3 M hydrogen peroxide solution was added. Subsequently, 15 µl of the homogenate was added, and the change in absorbance after 2 min was recorded. Absorbance was read at 240 nm using an ultraviolet/

visible (UV/Vis) spectrophotometer. Catalase activity was expressed as units/milligram protein using a molar extinction coefficient of 43.6 $M^{-1}/cm^{-1}$. All of the assays were performed in duplicate.

2.8. Statistical analysis

For comparison of the results among the experimental groups, two-way ANOVA was performed, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed on GraphPad Prism (version 5.00).

3. Results

3.1 Puberty installation, biochemical dosages and hormonal serum concentrations

The age of complete preputial separation was delayed in the groups exposed to rosuvastatin (Fig. 1), indicating delayed puberty installation in these experimental groups. Ascorbic acid was able to prevent delayed puberty in the groups co-exposed to rosuvastatin (Fig. 1). Total cholesterol and triglycerides serum concentrations were decreased in the rosuvastatin-treated groups (Fig. 2). FSH and LH concentrations were similar among the experimental groups (Fig. 2). Testosterone levels showed a gradual decrease between rosuvastatin-exposed groups when compared to the control group, however ascorbic acid was able to restore testosterone concentrations in the group co-exposed to the lower dose of rosuvastatin and improve, at least partially, testosterone levels in the group co-exposed to the higher dose of statin in relation to the group exposed to 10 mg of rosuvastatin alone (Fig. 2).

3.2. Testicular and epididymal morphology and morfometry

Testicular morphology of rosuvastatin-treated groups demonstrated diminished frequency of normal seminiferous tubules with increased rate of acidophilic cells such as spermatogonias and spermatocytes in the seminiferous epithelium in comparison to the control group (Table 1 and Fig. 3). Moreover, the group exposed to the higher dose of rosuvastatin also presented increased rate of germ cells in the lumen (Table 1 and Fig. 3). Ascorbic acid supplementation prevented these histopathological alterations observed in the groups exposed to both doses of rosuvastatin (Table 1 and Fig. 3).

The maturation degree of seminiferous epithelium was lower in the groups exposed to the statin when compared to the control group and it was also lower in the groups exposed to ascorbic acid in association with the statin (Table 1).

Rosuvastatin-exposed groups showed cribriform pattern with undifferentiated epithelium in the proximal epididymal cauda (Fig. 4). The animals treated with the higher dose of rosuvastatin also presented apparently lower diameter of the epididymal duct and lower sperm storage in the distal cauda (Fig. 4). Ascorbic acid supplementation might prevent these effects in the epididymis (Fig. 4).

### 3.3. Antioxidant activity

Testicular catalase activity was higher in the group treated with the lower dose of rosuvastatin in comparison with the other experimental groups (Fig. 5). Ascorbic acid supplementation prevented the increased testicular catalase activity in the group co-exposed to 3 mg of rosuvastatin and ascorbic acid (Fig. 5). GSH levels and lipid peroxidation in the testis were similar among experimental the groups (Fig. 5). Epididymal catalase activity, GSH levels and lipid peroxidation did not show statistical differences among the experimental groups (Fig. 6).

### 3.4. Final body weight and organ weights

Final body weight did not show significant differences among the experimental groups (Table 2). The reproductive and vital organ weights assessed in this study were not affected by the different experimental treatments (Table 2 and 3).

## 4. Discussion

This study is based on the attempt to prevent the reproductive adverse effects of rosuvastatin, used in therapeutic doses to prevent the cardiovascular risk in obese children, as previously reported [34]. Regardless of we have used normal, non-obese pre-pubertal *Wistar* rats, this study mimics the human exposure to the rosuvastatin, since statins show pleitropic effects that are beneficing many people that are using it [5,42].

Ascorbic acid has an antioxidant activity and may increase testosterone concentrations [28,43], thus it was used on the attempt to prevent the reproductive damage induced by rosuvastatin [34].

Rosuvastatin is a hypolipemiant agent and is capable to diminish total cholesterol, LDL-cholesterol and triglycerides levels in the blood [16,35]. Moreover, ascorbic acid supplementation is able to prevent HDL-cholesterol oxidation and increase HDL-cholesterol levels [44,45].

In this study, rosuvastatin-treated groups showed diminished total cholesterol and triglycerides levels when compared to the control group, indicating that the drug is capable to diminish these substances in the blood. The co-exposed groups presented decreased triglycerides levels but similar total cholesterol levels in comparison with control and ascorbic acid supplemented groups. This result is probably related to the increase of HDL-cholesterol that ascorbic acid may generate, thus leading to higher total cholesterol levels that are similar to the control and ascorbic acid supplemented groups.

Puberty installation begins with increased pulses of gonadotrophin releasing hormone (GnRH) in the hypothalamus, followed by an augmentation in the gonadotrophic hormones levels, such as FSH and LH, released by the pituitary and then, leading to increased sexual steroid hormones levels, such as testosterone secreted by the testis [23,46]. Androgens have an important role in the puberty installation and the acquisition of reproductive capability [23,46].

FSH and LH concentrations were not altered among the different experimental treatments in the protocol utilized. On the other hand, testosterone was gradually decreased in a dose-dependent manner in the groups exposed to rosuvastatin. Testosterone reduction observed in the rosuvastatin-exposed groups was partially prevented by ascorbic acid due to its crucial role in the regulation of gonadal steroidogenesis [43].

Increased testosterone concentrations are important to puberty installation, since testosterone allows preputial epithelium cornification, making preputial separation possible [47]. Preputial separation is a physical sign used as marker for puberty onset in male rats [37]. The age of puberty installation was delayed in animals exposed to rosuvastatin in both doses, although ascorbic acid was capable to avoid the delay in the age of puberty installation in the co-exposed groups, probably due to the increase in testosterone levels observed in these groups in comparison with the groups only exposed to rosuvastatin.

Statins are known to diminish the isoprenylation and the expression of CYP17A1, leading to decreased androgen synthesis, as well as shown in a previous study performed with theca-interstitial cells in vitro [48,49]. On the other hand, ascorbic acid is able to increase the activity of testicular steroidogenic enzymes 3β-HSD and 17β-HSD [50,51], StAR protein and P450scc, besides to raise the expression of LH receptor, thus resulting in augmented testosterone biosynthesis by Leydig cells [50].

Testosterone secreted by the Leydig cells are necessary to the maturation of the seminiferous epithelium and maintenance of spermatogenesis [52]. Rosuvastatin-exposed groups showed delayed degree of maturation of the germinal epithelium probably due to the decreased testosterone concentration presented by these experimental groups. Ascorbic acid supplementation was not sufficient to prevent the delay in the degree of maturation of the germinal epithelium in the co-exposed groups.

Increased levels of androgens are needful to germ cells surviving, since Sertoli cells need testosterone binding to its androgen receptors to secrete proteins related to development of meiotic cells [53,54]. When androgen concentrations are lower there may be increased oxidative stress and augmentation in the DNA damage [54], as well as, increased cell death of germ cells by apoptosis in the seminiferous tubules [54,55].

In addition, metabolites of mevalonate cascade, named intermediate isoprenoids, that are reduced due to the inhibition of the enzyme HMG-CoA reductase, play important roles in the organism, acting in many biological processes such as the maintenance of cell functions, proliferation, differentiation and cell death [8].

We found increased rate of acidofilic cells in the seminiferous epithelium and diminished frequency of normal seminiferous tubules that are associated with androgen depletion in the statin-exposed groups. We also showed increased rate of germ cells in the lumen in the group treated with the higher dose of rosuvastatin. Ascorbic acid partially prevented the increased rate of acidofilic cells in the testis and it might prevent the raised germ cell exfoliation in the lumen.

Epididymal epithelium differentiation in the postnatal life is related to increased androgen levels that lead to an augmentation in the expression of androgen receptors, which ones act stimulating epididymis differentiation [56]. Androgen depletion is associated with diminished androgen receptors expression in the epididymal cells and suggest compromising in the androgen-dependent mechanisms [22,34].

We demonstrated that prepubertal exposure to rosuvastatin may delay epididymis differentiation and that the animals presented cribriform pattern of the epithelium. Ascorbic acid supplemented groups did not show this histopathological alteration, indicating that, at least preventing partially androgen depletion, the epithelium may differentiate and develop similarly to the control group.

In mammals, the oxidative stress occurs due to an augmentation in the production of reactive oxygen species and nitrogen species that leads to higher levels of lipid peroxidation compounds that exceeds the antioxidant capability of cells [57]. The antioxidant system is formed by enzymes and small molecules soluble in water or lipids that includes glutathione, ascorbic acid, tocopherols and tocotrienols [57]. Catalase is an important antioxidant enzyme that acts decomposing hydrogen peroxide, which may provoke damage to the cells, to originate water and oxygen [41,57].

In this study, catalase activity in the testis was increased in the group exposed to the lower dose of rosuvastatin, probably due to the augmented production of hydrogen peroxide provoked by the statin at low doses. Ascorbic acid supplementation provided raised antioxidant capacity, thus in the group co-exposed to 3 mg of rosuvastatin and ascorbic acid it was not observed increased activity of this enzyme. Previous study also showed androgen depletion and increased catalase, superoxide dismutase and glutathione peroxidase activity in testicular tissue of rats exposed to different doses of functionalized multi-walled carbon nanotubes during puberty [58]. Epididymal catalase activity was not affected by the experimental treatments. In addition, GSH levels and lipid peroxidation were similar among the groups in the testis and epididymis.

Ascorbic acid seems to be most effective at the dose of 3 mg of rosuvastatin due to the restoration of serum testosterone concentrations that prevented the reproductive impairment provoked by rosuvastatin. At the higher dose of the statin, the amount of reproductive damage might be prevented by ascorbic acid due to the association of the improvement on testosterone levels in a critical period, such as puberty, and probably due to increased antioxidant response associated with other antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and glutathione s-transferase, as previously reported in different experimental protocols with ascorbic acid supplementation [59–61].

Ascorbic acid improved testosterone at the dose of 10 mg of rosuvastastin, making its androgen levels similar to the group that received only rosuvastatin at the dose of 3 mg. Considering that antioxidant status is accompanied by augmented levels of growth hormone (GH) and testosterone and that antioxidant status increases with the progression of pubertal development [62,63], we suppose that the groups only exposed to rosuvastatin may not exhibit their complete antioxidant capability, however ascorbic acid, as a potent antioxidant vitamin that represents up to 65% of antioxidant status on semen [29,64], may contribute on the maturation of antioxidant system and improve antioxidant status in the co-exposed groups, thus contributing to the restoration of the delayed puberty onset.

Final body weight provides substantial information about the health condition of the animals [65]. Moreover, reproductive organ weights gives us general information about reproductive parameters of the rats [65], as wells as, vital organ weights allow us to infer the general condition of organs that have a critical role on detoxification or vital importance to the animals. We observed no alterations on body weight and reproductive and vital organ weights among the experimental groups in this work.

5. Conclusion

Juvenile exposure to rosuvastatin delayed puberty installation, followed by delayed seminiferous epithelium maturation and delayed epididymal differentiation, besides to provoke germ cell death. These adverse effects are associated with androgen depletion observed in the statin-exposed animals. Ascorbic acid was capable to prevent partially the reproductive damage induced by rosuvastatin. We conclude that prepubertal exposure to rosuvastatin is a probable risk to reproductive development and supplementation with ascorbic acid is a possible simple form to prevent the reproductive adverse effects generated by rosuvastatin exposure.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgments

The authors are grateful to São Paulo Research Foundation (FAPESP) (Grant #013/22495-1), National Council for Scientific and Technological Development (CNPq) (Grant 308842/2013-8) and Coordination for the Improvement of Higher Education Personnel (CAPES) for their financial support and to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for excellent technical assistance.

8. References

[1] E.G. Jiménez, J.A. Ferre, Estatinas : Características y Efectos sobre el Control Lipídico en el Niño y Adolescente Obeso, Rev. Clínica Med. Fam. 4 (2011) 69–75.

[2] P.O. Kwiterovich, Recognition and management of dyslipidemia in children and adolescents, J. Clin. Endocrinol. Metab. 93 (2008) 4200–4209. doi:10.1210/jc.2008-1270.

[3] M.C.D.O. Izar, M.I.H. Fonseca, F.A.H. Fonseca, Dyslipidemias, Rev. Bras. Med. 68 (2011) 59–73. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L364430815.

[4] E.S. Istvan, J. Deisenhofer, Structural mechanism for statin inhibition of HMG-CoA reductase, Science (80-. ). 292 (2001) 1160–1164. doi:10.1126/science.1059344.

[5] V. Tandon, G. Bano, V. Khajuria, A. Parihar, S. Gupta, Pleiotropic effects of statins, Indian J. Pharmacol. 37 (2005) 77–85. doi:10.5772/23502.

[6] M. Endres, Statins: Potential new indications in inflammatory conditions, Atheroscler. Suppl. 7 (2006) 31–35. doi:10.1016/j.atherosclerosissup.2006.01.005.

[7] E. Istvan, Statin inhibition of HMG-CoA reductase: A 3-dimensional view, Atheroscler. Suppl. 4 (2003) 3–8. doi:10.1016/S1567-5688(03)00003-5.

[8] O. Adam, U. Laufs, Antioxidative effects of statins, Arch. Toxicol. 82 (2008) 885–892. doi:10.1007/s00204-008-0344-4.

[9] E.H. Corder, A.M. Saunders, W.J. Strittmatter, D.E. Schmechel, P.C. Gaskell, G.W. Small, A.D. Roses, J.L. Haines, M.A. Pericak-Vance, Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families., Science. 261 (1993) 921–923. doi:10.1126/science.8346443.

[10] V.L. Campo, I. Carvalho, Estatinas hipolipêmicas e novas tendências terapêuticas, Quim. Nova. 30 (2007) 425–430. doi:10.1590/S0100-40422007000200033.

[11] W. Feleszko, R. Zagozdzon, J. Gołąb, M. Jakóbisiak, Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice, Eur. J. Cancer. 34 (1998) 406–411. doi:10.1016/S0959-8049(97)10034-X.

[12] T. Tanaka, I. Tatsuno, D. Uchida, I. Moroo, H. Morio, S. Nakamura, Y. Noguchi, T. Yasuda, M. Kitagawa, Y. Saito, a Hirai, Geranylgeranyl-pyrophosphate, an isoprenoid of

mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition., J. Neurosci. 20 (2000) 2852–9. http://www.ncbi.nlm.nih.gov/pubmed/10751437.

[13] S.K. Lee, Y.S. Kim, Phosphorylation of eIF2α attenuates statin-induced apoptosis by inhibiting the stabilization and translocation of p53 to the mitochondria, Int. J. Oncol. 42 (2013) 810–816. doi:10.3892/ijo.2013.1792.

[14] X.-F. Qi, L. Zheng, K.-J. Lee, D.-H. Kim, C.-S. Kim, D.-Q. Cai, Z. Wu, J.-W. Qin, Y.-H. Yu, S.-K. Kim, HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway., Cell Death Dis. 4 (2013) e518. doi:10.1038/cddis.2013.44.

[15] G.A. Holdgate, W.H.J. Ward, F. McTaggart, Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin., Biochem. Soc. Trans. 31 (2003) 528–531. doi:10.1042/BST0310528.

[16] F. McTaggart, Comparative pharmacology of rosuvastatin, Atheroscler. Suppl. 4 (2003) 9–14. doi:10.1016/S1567-5688(03)00004-7.

[17] T.E. Stoker, S.C. Laws, D.L. Guidici, R.L. Cooper, The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function, Toxicol. Sci. 58 (2000) 50–59. doi:10.1093/toxsci/58.1.50.

[18] W. Zawatski, M.M. Lee, Male pubertal development: Are endocrine-disrupting compounds shifting the norms?, J. Endocrinol. 218 (2013) 1–12. doi:10.1530/JOE-12-0449.

[19] A.P.A. Favareto, C.D.B. Fernandez, D.A.F. da Silva, J.A. Anselmo-Franci, W.D.G. Kempinas, Persistent Impairment of Testicular Histology and Sperm Motility in Adult Rats Treated with Cisplatin at Peri-Puberty, Basic Clin. Pharmacol. Toxicol. 109 (2011) 85–96. doi:10.1111/j.1742-7843.2011.00688.x.

[20] Johnson L., T.H.J. Welsh, C.E. Wilker, Anatomy and physiology of the male reproductive system and potential targets of toxicants., in: K. Boekleheide, R. Chapin, C. Hoyer, P. Harris (Eds.), Compr. Toxicol., Pergamon, New York, 1997: pp. 5–61.

[21] C.R. Blystone, C.S. Lambright, J. Furr, V.S. Wilson, L.E. Gray, Iprodione delays male rat pubertal development, reduces serum testosterone levels, and decreases ex vivo testicular

testosterone production, Toxicol. Lett. 174 (2007) 74–81. doi:10.1016/j.toxlet.2007.08.010.

[22] J.E. Perobelli, M.T.C.C. Patrão, C.D.B. Fernandez, M. Sanabria, G.R. Klinefelter, M.C.W. Avellar, W.D.G. Kempinas, Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis, Reprod. Toxicol. 38 (2013) 65–71. doi:10.1016/j.reprotox.2013.03.004.

[23] A. Mantovani, A. Fucic, Puberty dysregulation and increased risk of disease in adult life: Possible modes of action, Reprod. Toxicol. 44 (2014) 15–22. doi:10.1016/j.reprotox.2013.06.002.

[24] G. Corsetti, A. Stacchiotti, L. Tedesco, G. D'Antona, E. Pasini, F.S. Dioguardi, E. Nisoli, R. Rezzani, Essential amino acid supplementation decreases liver damage induced by chronic ethanol consumption in rats., Int. J. Immunopathol. Pharmacol. 24 (2011) 611–619.

[25] M. Sooriyaarachchi, A. Narendran, J. Gailer, The effect of sodium thiosulfate on the metabolism of cis-platin in human plasma in vitro, Metallomics. 4 (2012) 960–967. doi:10.1039/c2mt20076g.

[26] P.K. Mukhopadhyay, A. Dey, S. Mukherjee, N.K. Pradhan, The effect of coadministration of alpha-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats, J. Basic Clin. Physiol. Pharmacol. 24 (2013) 245–253. doi:10.1515/jbcpp-2012-0039.

[27] D. Pandir, O. Kara, M. Kara, Protective effect of bilberry (Vaccinium myrtillus L.) on cisplatin induced ovarian damage in rat, Cytotechnology. 66 (2014) 677–685. doi:10.1007/s10616-013-9621-z.

[28] M. Sönmez, G. Turk, A. Yuce, The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats, Theriogenology. 63 (2005) 2063–2072. doi:10.1016/j.theriogenology.2004.10.003.

[29] G.S.A. Fernandes, C.D.B. Fernandez, K.E. Campos, D.C. Damasceno, J.A. Anselmo-Franci, W.D.G. Kempinas, Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats., Reprod. Biol. Endocrinol. 100 (2011) 1–9. doi:10.1186/1477-7827-9-100.

[30] G.S.A. Fernandes, D.C.C. Gerardin, T.A. Assumpção, K.E. Campos, D.C. Damasceno,

O.C.M. Pereira, W.D.G. Kempinas, Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats?, Pharmacol. Reports. 63 (2011) 983–991.

[31] A. Agarwal, S.A. Prabhakaran, T.M. Said, Prevention of Oxidative Stress Injury to Sperm, J. Androl. 26 (2005) 654–660. doi:10.2164/jandrol.05016.

[32] B. Eskenazi, S.A. Kidd, A.R. Marks, E. Sloter, G. Block, A.J. Wyrobek, Antioxidant intake is associated with semen quality in healthy men, Hum. Reprod. 20 (2005) 1006–1012. doi:10.1093/humrep/deh725.

[33] B. Shrilatha, Muralidhara, Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences, Reprod. Toxicol. 23 (2007) 578–587. doi:10.1016/j.reprotox.2007.02.001.

[34] G.A.A. Leite, J. de L. Rosa, M. Sanabria, M.M. Cavariani, J.A.A. Franci, P.F.F. Pinheiro, W.D.G. Kempinas, Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty, Reprod. Toxicol. 44 (2014) 93–103. doi:10.1016/j.reprotox.2014.01.004.

[35] C.J. Vaughan, A.M. Gotto Jr, Update on Statins: 2003, Circulation. 110 (2004) 886–892. doi:10.1161/01.CIR.0000139312.10076.BA.

[36] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited., FASEB J. 22 (2008) 659–661. doi:10.1096/fj.07-9574LSF.

[37] R.M. Parker, Testing for reproductive toxicity., in: R.D. Hood (Ed.), Dev. Reprod. Toxicol. A Pract. Approach, Third edit, CRC Press, New York, 2006: pp. 472–474.

[38] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254. doi:10.1016/0003-2697(76)90527-3.

[39] H.H. Draper, M. Hadley, Malondialdehyde determination as index of lipid peroxidation, Methods Enzymol. 186 (1990) 421–431. doi:10.1016/0076-6879(90)86135-I.

[40] G.L. Ellman, Tissue Sulfhydryl Groups, Arch. Biochem. Biophys. 82 (1959) 70–77.

[41] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126. doi:10.1016/S0076-6879(84)05016-3.

[42] A. Park, Statins have few side effects, but should more people be taking them?, Time. (2013). http://healthland.time.com/2013/07/10/statins-have-few-side-effects-but-should-

more-people-be-taking-them/.

[43] U.A. Okon, I.I. Utuk, Ascorbic acid treatment elevates follicle stimulating hormone and testosterone plasma levels and enhances sperm quality in albino Wistar rats., Niger. Med. J. 57 (2016) 31–36.

[44] M. Siavash, M. Amini, Vitamin C may have similar beneficial effects to Gemfibrozil on serum high - density lipoprotein - cholesterol in type 2 diabetic patients, J. Res. Pharm. Pract. 3 (2014) 77–82.

[45] R.J. Hillstrom, A.K. Yacapin-ammons, S.M. Lynch, Vitamin C Inhibits Lipid Oxidation in Human HDL, Biochem. Mol. Actions Nutr. 10 (2003) 3047–3051.

[46] M.M. Fisher, E.A. Eugster, What is in our environment that effects puberty ?, Reprod. Toxicol. 44 (2014) 7–14.

[47] W.R. Lyons, I. Berlin, S.O.L. Friedlander, Cornification of balano-preputial epithelium in normal rats and in castrated rats treated with testosterone propionate, Endocrinology. 31 (1942) 659–663.

[48] I. Ortega, J.A. Villanueva, D.H. Wong, A.B. Cress, A. Sokalska, S.D. Stanley, A.J. Duleba, Resveratrol potentiates effects of simvastatin on inhibition of rat ovarian theca-interstitial cells steroidogenesis., J. Ovarian Res. 7 (2014) 21. doi:10.1186/1757-2215-7-21.

[49] I. Ortega, A.B. Cress, D.H. Wong, J. a Villanueva, A. Sokalska, B.C. Moeller, S.D. Stanley, A.J. Duleba, Simvastatin reduces steroidogenesis by inhibiting Cyp17a1 gene expression in rat ovarian theca-interstitial cells., Biol. Reprod. 86 (2012) 1–9. doi:10.1095/biolreprod.111.094714.

[50] H. Radhakrishnakartha, A.P. Appu, M. Indira, Ascorbic acid supplementation enhances recovery from ethanol induced inhibition of Leydig cell steroidogenesis than abstention in male guinea pigs, Eur. J. Pharmacol. 723 (2014) 73–79. doi:10.1016/j.ejphar.2013.12.010.

[51] R. Harikrishnan, P.A. Abhilash, S. Syam Das, P. Prathibha, S. Rejitha, F. John, S. Kavitha, M. Indira, Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male guinea pigs., Br. J. Nutr. 110 (2013) 689–98. doi:10.1017/S0007114512005739.

[52] D.A. Adamopoulos, P. Vassilopoulos, L. Kontogeorgos, N. Kapolla, Germinal epithelium changes in sexually immature rabbits treated with intratesticular testosterone implants, Andrologia. 22 (1990) 557–565.

[53] K. De Gendt, J. V Swinnen, P.T.K. Saunders, L. Schoonjans, M. Dewerchin, A. Devos, K. Tan, N. Atanassova, F. Claessens, C. Lécureuil, W. Heyns, P. Carmeliet, F. Guillou, R.M. Sharpe, G. Verhoeven, A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis., Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 1327–32. doi:10.1073/pnas.0308114100.

[54] P.G. Stanton, P. Sluka, C.F.H. Foo, A.N. Stephens, A.I. Smith, R.I. Mclachlan, L.O. Donnell, Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation: Impact on meiotic cells, PLoS One. 7 (2012) 1–19. doi:10.1371/journal.pone.0041718.

[55] Y. Lue, A.P.S. Hikim, R.S. Swerdloff, P. Im, K.S. Taing, T.A.N. Bui, A. Leung, C. Wang, Single Exposure to Heat Induces Stage-Specific Germ Cell Apoptosis in Rats : Role of Intratesticular Testosterone on Stage Specificity, Endocrinology. 140 (1999) 1709–1717.

[56] L. You, M. Sar, Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats., Endocrine. 9 (1998) 253–61. doi:10.1385/ENDO:9:3:253.

[57] P.-A. Mouthuy, S.J.B. Snelling, S.G. Dakin, L. Milković, A.C. Gašparović, A.J. Carr, N. Žarković, Biocompatibility of implantable materials: An oxidative stress viewpoint, Biomaterials. 109 (2016) 55–68. doi:10.1016/j.biomaterials.2016.09.010.

[58] E.O. Farombi, I.A. Adedara, G.E. Forcados, O.O. Anao, A. Agbowo, A.K. Patlolla, Responses of testis, epididymis and sperm of pubertal rats exposed to functionalized multiwalled carbon nanotubes, Environ. Toxicol. 31 (2016) 543–51. doi:10.1002/tox.22067.

[59] G. Jelodar, S. Nazifi, a Akbari, The prophylactic effect of vitamin C on induced oxidative stress in rat testis following exposure to 900 MHz radio frequency wave generated by a BTS antenna model, Electromagn Biol Med. 32 (2013) 409–416. doi:10.3109/15368378.2012.735208.

[60] E.A. Ahmed, H.M. Omar, S.K.A. elghaffar, S.M.M. Ragb, A.Y. Nasser, The antioxidant activity of Vitamin C, DPPD and l-cysteine against Cisplatin-induced testicular oxidative damage in rats, Food Chem. Toxicol. 49 (2011) 1115–1121. doi:10.1016/j.fct.2011.02.002.

[61] M. Mohasseb, S. Ebied, M.A.H. Yehia, N. Hussein, Testicular oxidative damage and role of combined antioxidant supplementation in experimental diabetic rats, J. Physiol.

Biochem. 67 (2011) 185–194. doi:10.1007/s13105-010-0062-2.

[62] G. Paltoglou, I.G. Fatouros, G. Valsamakis, M. Schoina, A. Avloniti, A. Chatzinikolaou, A. Kambas, D. Draganidis, A. Mantzou, M. Papagianni, C. Kanaka-Gantenbein, G.P. Chrousos, G. Mastorakos, Antioxidation improves in puberty in normal weight and obese boys, in positive association with exercise-stimulated growth hormone secretion, Pediatr. Res. 78 (2015) 158–164. doi:10.1038/pr.2015.85.

[63] A. Avloniti, A. Chatzinikolaou, C. Deli, D. Vlachopoulos, L. Gracia-Marco, D. Leontsini, D. Draganidis, A. Jamurtas, G. Mastorakos, I. Fatouros, Exercise-induced oxidative stress responses in the pediatric population, Antioxidants. 6 (2017) 6. doi:10.3390/antiox6010006.

[64] K. Makker, A. Agarwal, R. Sharma, Oxidative stress & male infertility., Indian J. Med. Res. (2009) 357–367.

[65] E.D. Clegg, S.D. Perreault, G.R. Klinefelter, Assessment of male reproductive toxicology, in: A.W. Hayes (Ed.), Princ. Methods Toxicol., 4 th, Taylor & Francis, Philadelphia, 2001: pp. 1263–1299.

**Legends of figures**

**Fig. 1.** Age of complete preputial separation. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$.

**Fig. 2.** Serum biochemical measurements (mg/dl) and hormonal concentrations (ng/ml) in male rats at 53 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$.

**Fig. 3.** Photomicrography of testicular sections from the experimental groups on PND53. Observe the presence of acidophilic germ cells (arrows) that include spermatogonias and spermatocytes in the seminiferous epithelium and the presence of germ cells in the lumen (arrowhead). Hematoxylin and Eosin (HE). Scale bar = 100µm or 50µm.

**Fig. 4.** Photomicrography of epididymal sections from the experimental groups on PND53. Observe the presence of cribriform epithelium (arrows) in the rosuvastatin-exposed groups. Note

an apparent smaller diameter in the epididymal duct in the distal cauda of the group treated with the higher dose of rosuvastatin. (asterisks). Hematoxylin and Eosin (HE). Scale bar = 100µm.

**Fig. 5.** Lipid peroxidation, GSH levels and catalase activity in the testis from the different experimental groups on PND 53. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$.

**Fig. 6.** Lipid peroxidation, GSH levels and catalase activity in the epididymis from the different experimental groups on PND 53. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p > 0.05$.

**Table 1.** Maturation degree of seminiferous epithelium and testicular histopathological evaluation from experimental groups on PND 53.

| | Experimental Groups (n=9 or 10/group) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| [1]Maturation degree of seminiferous epithelium | $4.55 \pm 0.04^{a}$ | $4.35 \pm 0.03^{b}$ | $4.35 \pm 0.03^{b}$ | $4.61 \pm 0.03^{a}$ | $4.36 \pm 0.04^{b}$ | $4.41 \pm 0.05^{b}$ |
| [2]Normal seminiferous tubules (%) | $98.50\ (97.50 - 99.00)^{a}$ | $95.00\ (93.00 - 95.50)^{b}$ | $92.25\ (91.38 - 95.00)^{b}$ | $98.50\ (98.00 - 99.00)^{a}$ | $97.50\ (96.50 - 98.00)^{a}$ | $97.00\ (95.50 - 97.50)^{a}$ |
| [2]Seminiferous tubules with acidophilic cells (%) | $0.50\ (0 - 1.00)^{a}$ | $4.00\ (3.25 - 6.00)^{b}$ | $2.00\ (1.37 - 4.12)^{b}$ | $0\ (0 - 0.87)^{a}$ | $2.00\ (0 - 2.50)^{ab}$ | $2.00\ (1.62 - 3.00)^{ab}$ |
| [2]Seminiferous tubules with germ cells in the lumen (%) | $0.50\ (0 - 1.00)^{a}$ | $0.50\ (0 - 1.00)^{a}$ | $2.25\ (0.87 - 6.12)^{b}$ | $0.50\ (0.50 - 1.00)^{a}$ | $0.50\ (0 - 0.50)^{a}$ | $0.50\ (0 - 0.50)^{a}$ |

Values expressed as mean ± SEM[1] or median and interquartile intervals[2]. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Table 2.** Body weight and reproductive organ weights from the experimental groups on PND 53.

| Parameters (n = 10) | Control | 3 mg | 10 mg | AA | 3 mg + AA | 10 mg + AA |
|---|---|---|---|---|---|---|
| Body weight (g) | 230.20 ± 6.10 | 231.50 ± 5.19 | 225.50 ± 4.94 | 218.00 ± 4.43 | 212.70 ± 5.59 | 207.9 ± 3.45 |
| Testis (g) | 1.18 ± 0.05 | 1.19 ± 0.03 | 1.17 ± 0.04 | 1.18 ± 0.03 | 1.14 ± 0.04 | 1.14 ± 0.04 |
| Testis (g/100g BW) | 0.52 ± 0.02 | 0.53 ± 0.01 | 0.51 ± 0.02 | 0.54 ± 0.01 | 0.55 ± 0.01 | 0.55 ± 0.01 |
| Epididymis (mg) | 172.70 ± 7.64 | 178.00 ± 9.09 | 174.80 ± 6.53 | 167.40 ± 4.36 | 163.6 ± 7.65 | 172.00 ± 8.12 |
| Epididymis (mg/100g BW) | 76.47 ± 3.63 | 79.54 ± 2.65 | 77.19 ± 2.92 | 76.82 ± 2.32 | 78.35 ± 2.62 | 83.68 ± 3.23 |
| Vas deferens (mg) | 43.52 ± 2.92 | 42.96 ± 2.42 | 44.57 ± 1.61 | 42.28 ± 1.64 | 41.39 ± 2.77 | 43.89 ± 3.90 |
| Vas deferens (mg/100g BW) | 19.43 ± 1.92 | 19.54 ± 0.81 | 19.84 ± 1.00 | 19.44 ± 1.02 | 19.76 ± 1.09 | 21.59 ± 2.05 |
| Prostate (mg) | 133.10 ± 12.64 | 138.00 ± 11.44 | 140.00 ± 11.72 | 140.60 ± 6.44 | 120.70 ± 14.95 | 121.00 ± 20.11 |
| Prostate (mg/100g BW) | 54.52 ± 3.84 | 62.98 ± 4.36 | 60.14 ± 3.58 | 63.84 ± 1.68 | 55.70 ± 5.94 | 57.42 ± 8.17 |
| Full seminal gland (mg) | 212.70 ± 29.64 | 200.40 ± 29.61 | 215.50 ± 24.60 | 197.10 ± 18.97 | 156.40 ± 22.89 | 148.20 ± 18.13 |
| Full seminal gland (mg/100g BW) | 84.17 ± 9.93 | 88.33 ± 11.42 | 91.91 ±8.50 | 88.73 ± 7.15 | 71.80 ± 8.73 | 71.68 ± 8.50 |
| Empty seminal gland (mg) | 95.80 ± 8.90 | 86.67 ± 7.84 | 103.30 ± 9.94 | 94.93 ± 7.44 | 76.31 ± 8.82 | 77.05 ± 7.03 |
| Empty seminal gland (mg/100g BW) | 39.35 ± 2.55 | 38.52 ± 2.89 | 44.76 ± 3.84 | 43.01 ± 2.90 | 35.35 ± 3.31 | 37.58 ± 3.32 |

Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p > 0.05$. BW = Body weight.

**Table 3.** Vital organ weights from the experimental groups on PND 53.

| Parameters (n = 10) | Control | 3 mg | 10 mg | AA | 3 mg + AA | 10 mg + AA |
|---|---|---|---|---|---|---|
| Pituitary (mg) | 6.15 ± 0.39 | 5.73 ± 0.39 | 6.65 ± 0.36 | 6.63 ± 0.33 | 6.33 ± 0.32 | 5.95 ± 0.38 |
| Pituitary (mg/100g PC) | 2.70 ± 0.13 | 2.67 ± 0.12 | 2.91 ± 0.13 | 3.02 ± 0.13 | 3.05 ± 0.15 | 2.86 ± 0.10 |
| Thyroid (mg) | 13.15 ± 1.00 | 13.47 ± 1.93 | 12.68 ± 1.40 | 12.80 ± 2.14 | 14.86 ± 1.35 | 11.65 ± 1.50 |
| Thyroid (mg/100g PC) | 6.21 ± 0.65 | 5.39 ± 0.39 | 5.70 ± 0.70 | 6.96 ± 0.14 | 7.42 ± 0.97 | 5.73 ± 0.77 |
| Liver (g) | 14.50 ± 0.80 | 14.38 ± 0.63 | 14.62 ± 0.77 | 13.84 ± 0.55 | 13.41 ± 0.70 | 13.12 ± 0.47 |
| Liver (g/100g PC) | 6.31 ± 0.15 | 6.37 ± 0.20 | 6.34 ± 0.13 | 6.30 ± 0.14 | 6.41 ± 0.18 | 6.37 ± 0.10 |
| Adrenal (mg) | 21.05 ± 0.75 | 20.80 ± 1.07 | 19.15 ± 1.17 | 21.56 ± 1.20 | 20.85 ± 0.93 | 19.68 ± 1.52 |
| Adrenal (mg/100g PC) | 9.38 ± 0.36 | 9.47 ± 0.47 | 8.41 ± 0.41 | 9.83 ± 0.45 | 10.40 ± 0.55 | 9.44 ± 0.60 |
| Kidney (g) | 1.07 ± 0.05 | 1.05 ± 0.04 | 1.09 ± 0.04 | 1.04 ± 0.04 | 0.99 ± 0.05 | 0.97 ± 0.04 |
| Kidney (g/100g PC) | 0.47 ± 0.01 | 0.46 ± 0.01 | 0.48 ± 0.01 | 0.48 ± 0.01 | 0.47 ± 0.01 | 0.47 ± 0.01 |
| Brain (g) | 1.86 ± 0.02 | 1.82 ± 0.04 | 1.84 ± 0.02 | 1.88 ± 0.02 | 1.82 ± 0.02 | 1.83 ± 0.03 |
| Brain (g/100g PC) | 0.85 ± 0.05 | 0.83 ± 0.02 | 0.82 ± 0.03 | 0.86 ± 0.02 | 0.89 ± 0.04 | 0.90 ±0.03 |

Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p > 0.05$. BW = Body weight.

**Figure 1.**

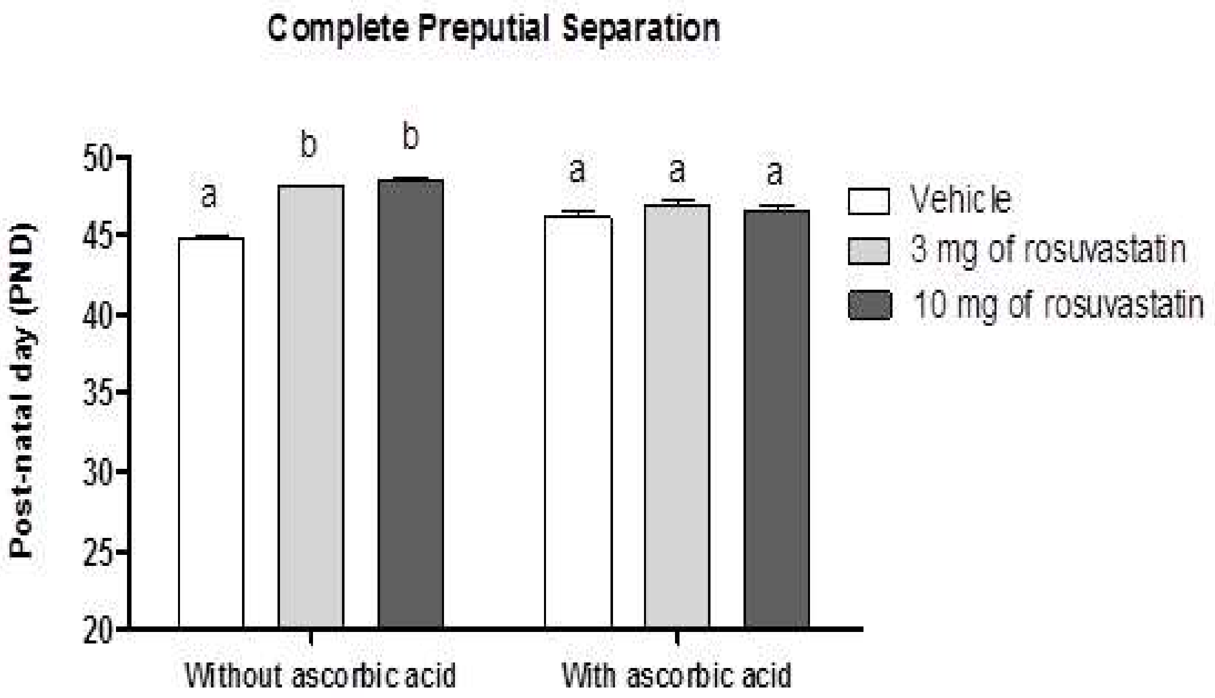
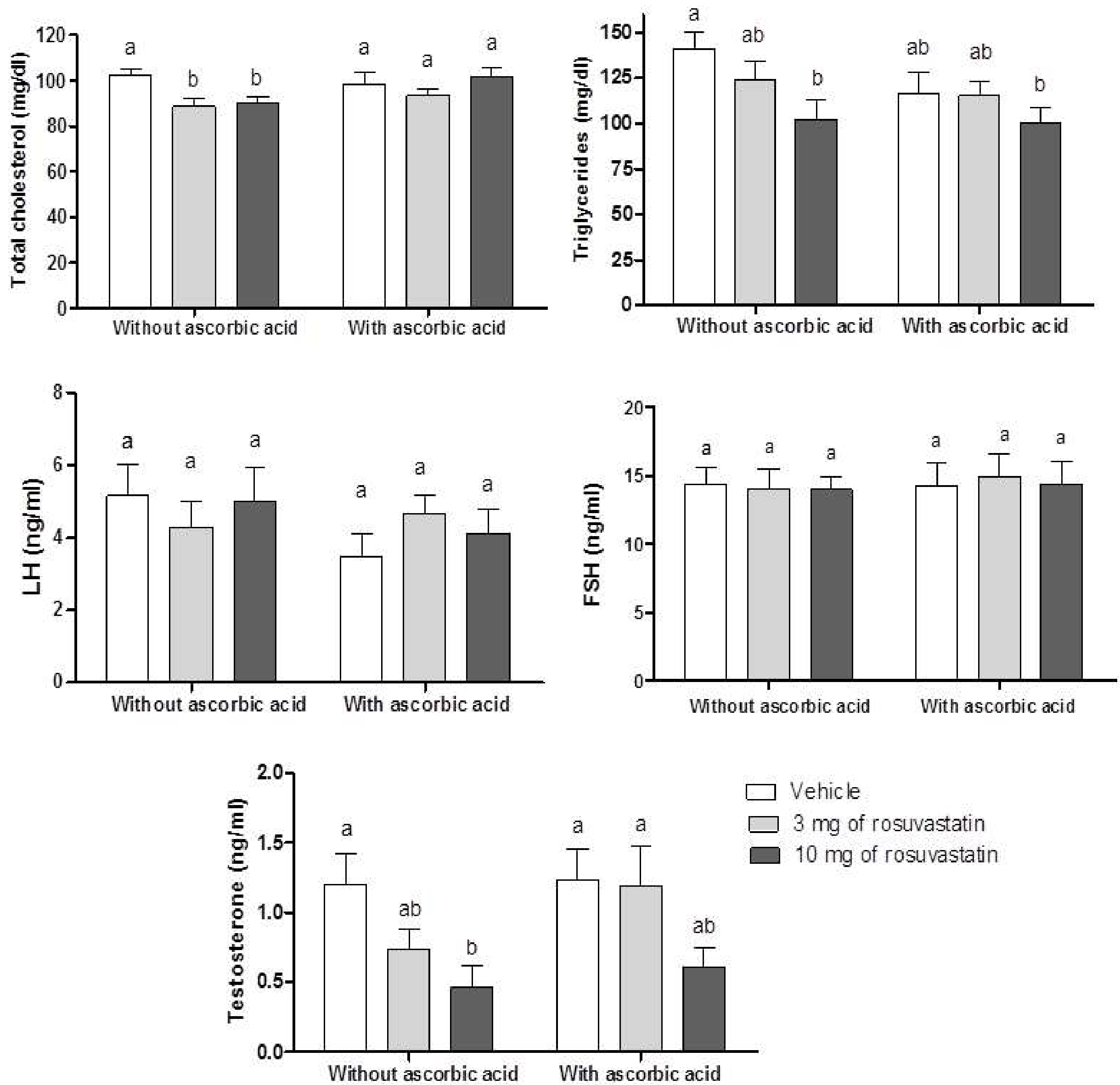

Complete Preputial Separation
Post-natal day (PND)
50
45
40
35
30
25
20
a
b
b
a
a
a
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin
Without ascorbic acid
With ascorbic acid

**Figure 2.**



Total cholesterol (mg/dl)
0
20
40
60
80
100
120
a
b
b
a
a
a
Without ascorbic acid
With ascorbic acid
Triglycerides (mg/dl)
0
25
50
75
100
125
150
a
ab
b
ab
ab
b
Without ascorbic acid
With ascorbic acid
LH (ng/ml)
0
2
4
6
8
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
FSH (ng/ml)
0
5
10
15
20
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Testosterone (ng/ml)
0.0
0.5
1.0
1.5
2.0
a
ab
b
a
a
ab
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 3.**

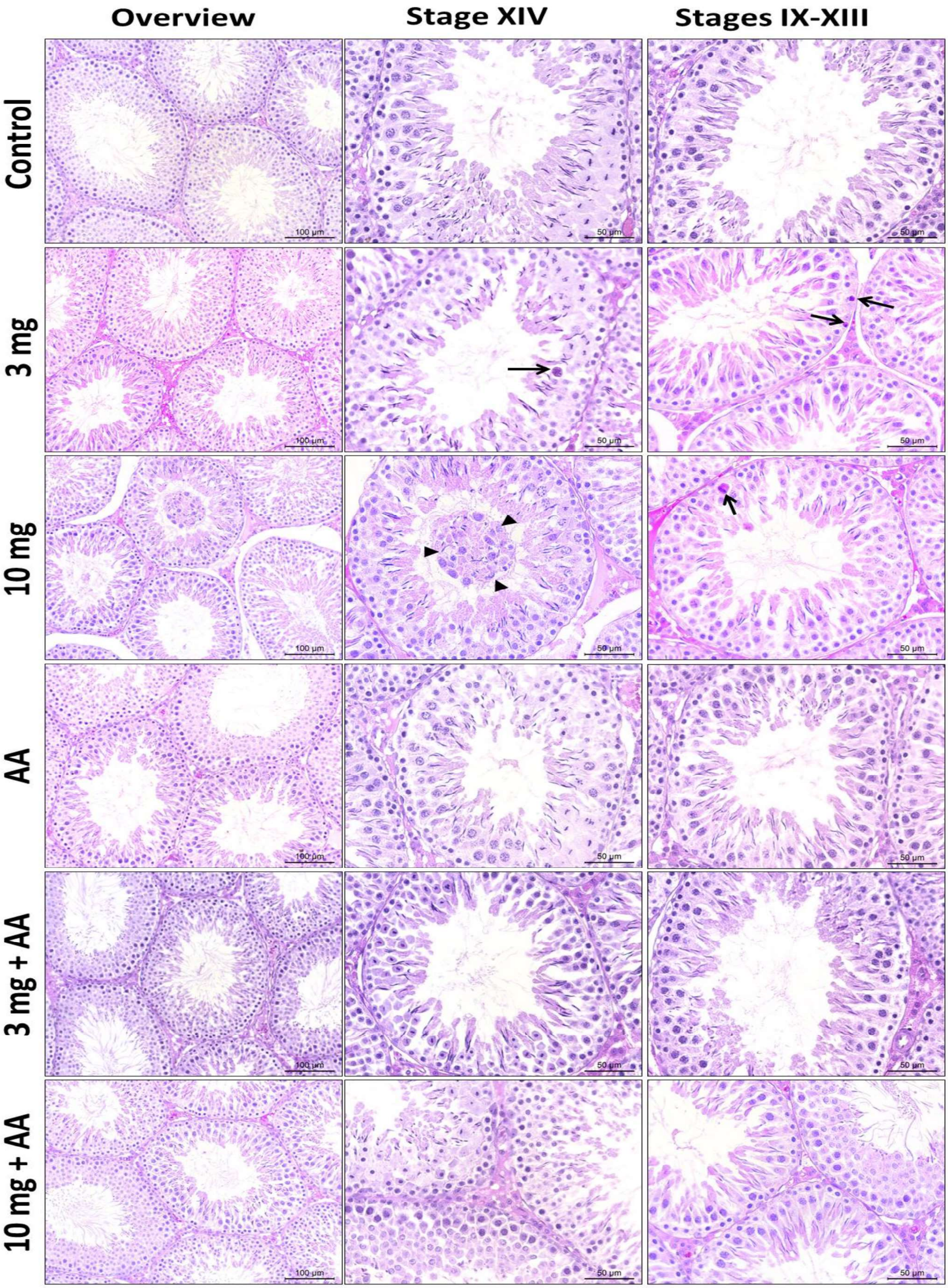

Overview
Stage XIV
Stages IX-XIII
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
100 µm
50 µm

Figure 4.

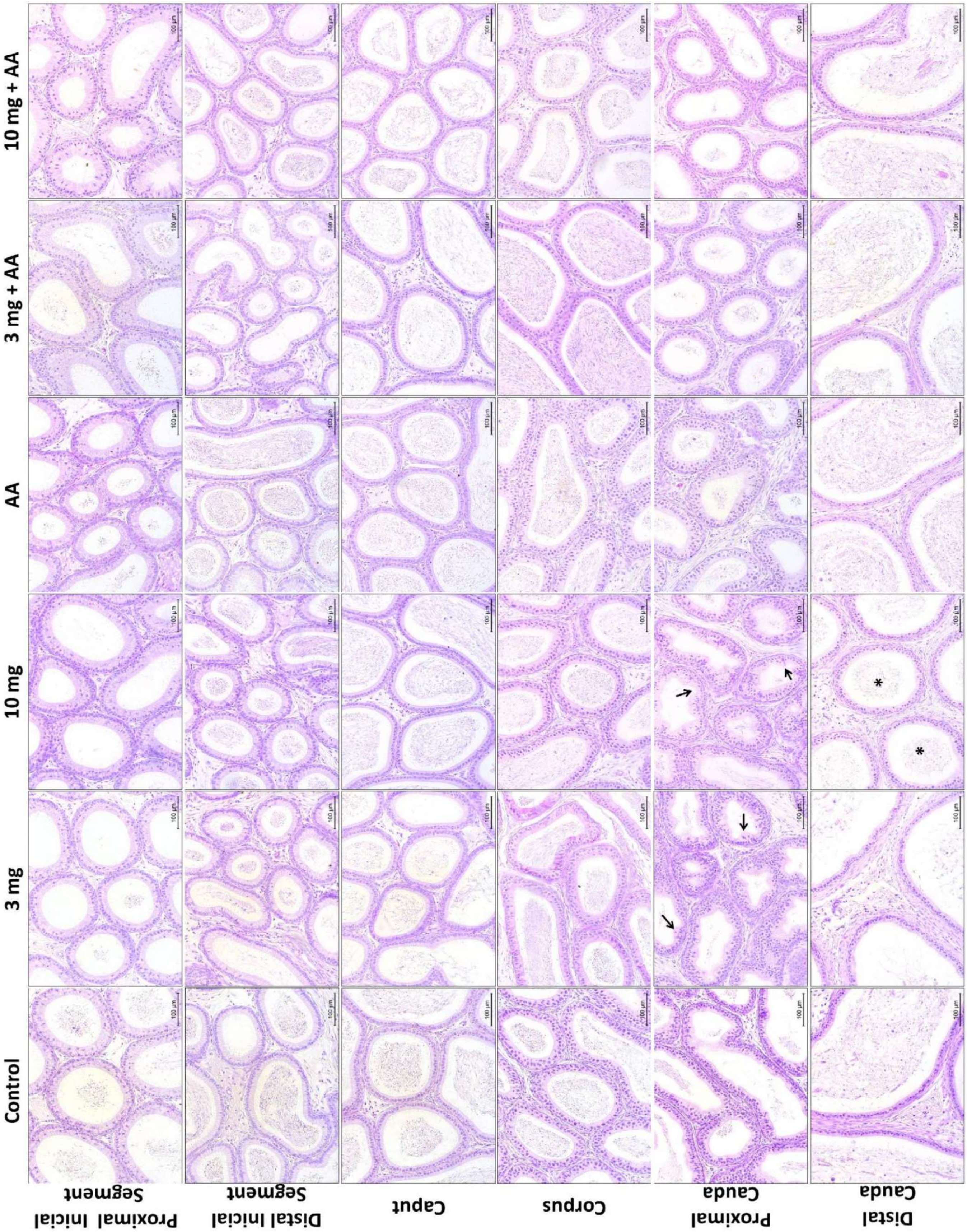
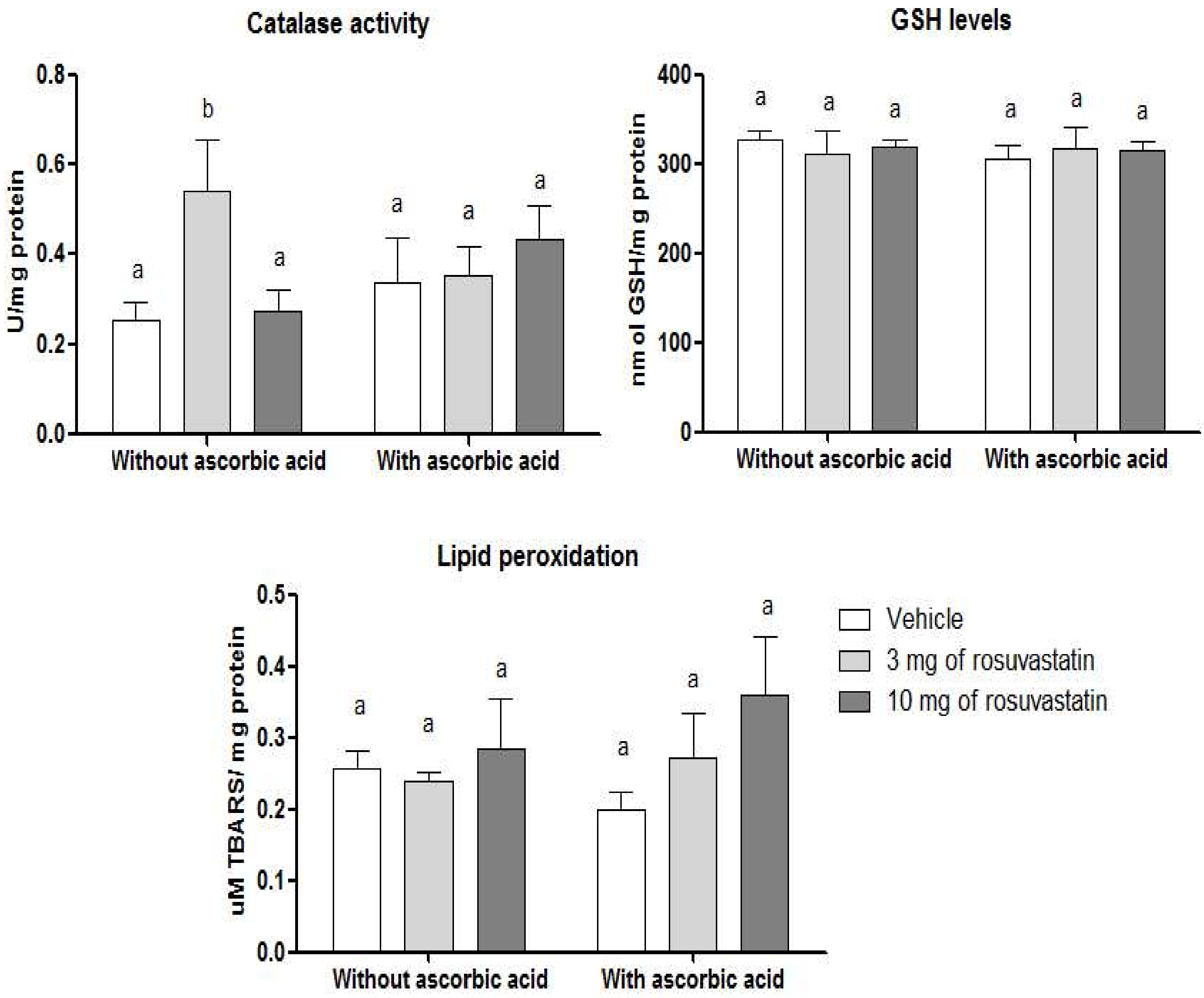
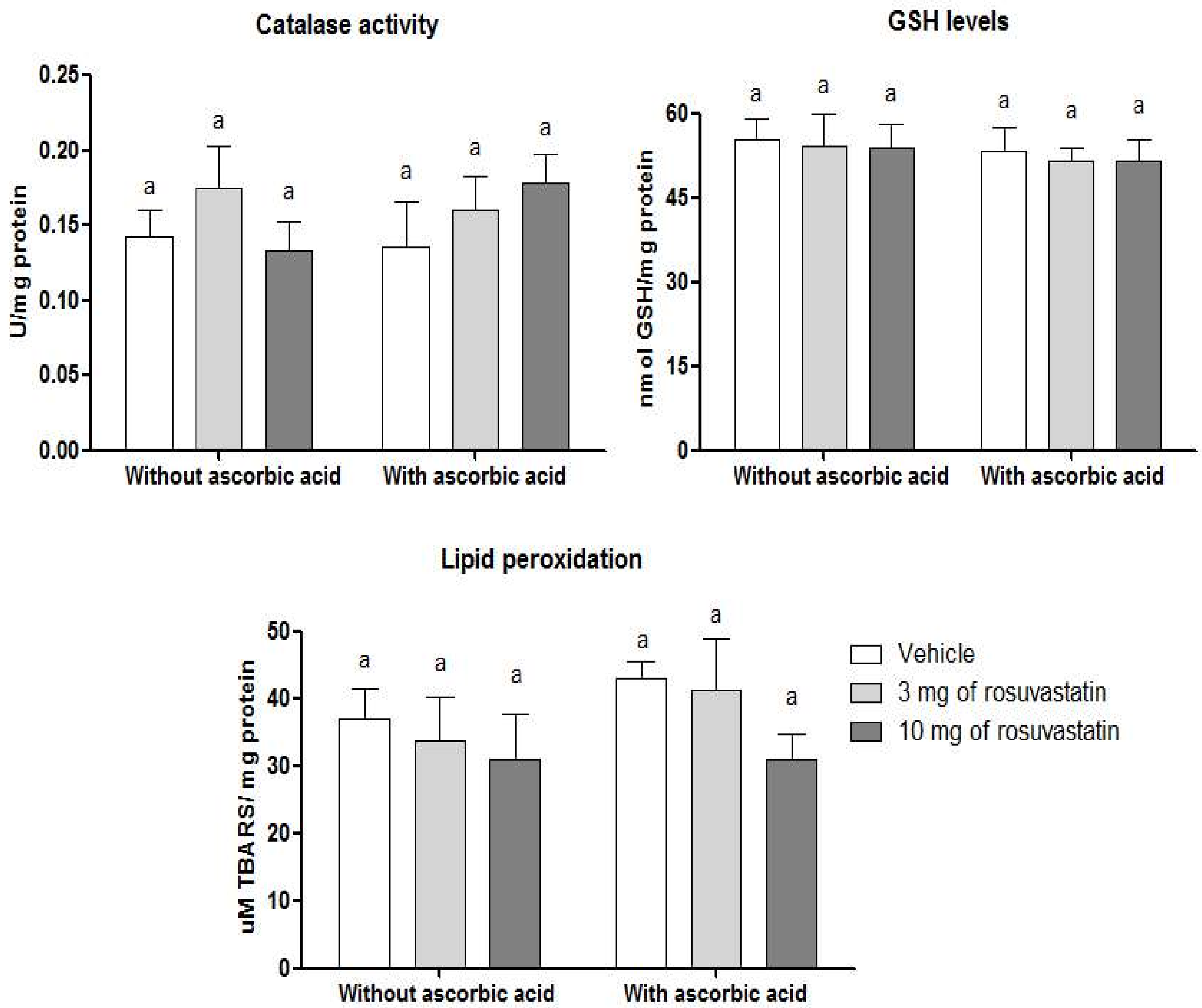

Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
Proximal Inicial Segment
Distal Inicial Segment
Caput
Corpus
Proximal Cauda
Distal Cauda

**Figure 5.**



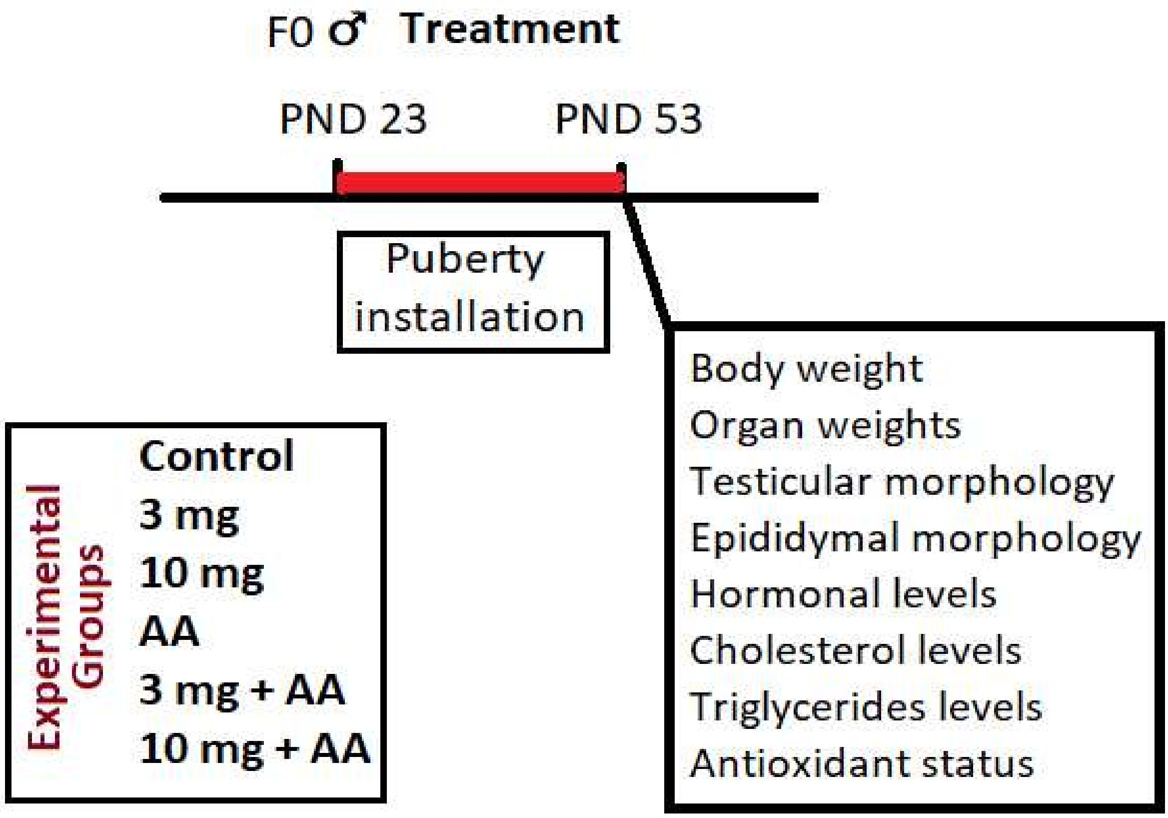
Catalase activity
U/mg protein
0.0
0.2
0.4
0.6
0.8
a
b
a
a
a
a
Without ascorbic acid
With ascorbic acid
GSH levels
nmol GSH/mg protein
0
100
200
300
400
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Lipid peroxidation
uM TBARS/ mg protein
0.0
0.1
0.2
0.3
0.4
0.5
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 6.**


Catalase activity
U/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
a
Without ascorbic acid
With ascorbic acid
GSH levels
nmol GSH/mg protein
60
45
30
15
0
Lipid peroxidation
uM TBARS/ mg protein
50
40
30
20
10
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Supplementary Material**

**Figure 1.** Description of the experimental design.



# Capítulo 2

**Manuscrito II**

O segundo manuscrito é intitulado **"Vitamin C partially prevents reproductive damage in adult male rats exposed to rosuvastatin during prepuberty"** de autoria de Gabriel Adan Araújo Leite, Thamiris Moreira Figueiredo, Tainá Louise Pacheco, Marciana Sanabria, Patrícia Villela e Silva, Fábio Henrique Fernandes e Wilma De Grava Kempinas, publicado na revista Food and Chemical Toxicology, volume 109, 1ª parte da 11ª edição, páginas 272-283, 2017. Elsevier, ISSN: 0278-6915. Fator de impacto: 3,778.

Food and Chemical Toxicology 109 (2017) 272–283

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Vitamin C partially prevents reproductive damage in adult male rats exposed to rosuvastatin during prepuberty

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ARTICLE INFO

*Article history:*
Received 16 January 2017
Received in revised form
19 August 2017
Accepted 2 September 2017
Available online 6 September 2017

*Keywords:*
Ascorbic acid
Rosuvastatin
Male reproduction
Toxicology

ABSTRACT

Pediatric obesity is closely associated with dyslipidemias and environmental factors, such as diet and lack of physical exercises, which may alter lipid profile in children. Rosuvastatin decreases serum total cholesterol and triglycerides concentrations. Vitamin C (ascorbic acid) plays an important role on sperm integrity and fertility. Juvenile male rats were distributed into six experimental groups that received saline solution 0.9%, 3 or 10 mg/kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 3 or 10 mg/kg/day of rosuvastatin co-administered with 150 mg/day of ascorbic acid from PND23 until PND53 and then the rats were maintained until sexual maturity. Rosuvastatin-exposed groups showed lower sperm quality, androgen depletion and germ cell death. Ascorbic acid was capable to prevent partially the reproductive adverse effects provoked by rosuvastatin. In conclusion, prepubertal exposure to rosuvastatin provokes long-term reproductive damages at sexual maturity and ascorbic acid supplementation at prepuberty may be a preventive mode against these reproductive adverse effects.



## 1. Introduction

Obesity, one of the greatest emerging problems of public health, has been affecting adults, children and adolescents (Jiménez and Ferre, 2011). Moreover, obesity is not only a common metabolic and nutritional disorder in developed countries but is also affecting underdeveloped countries (Jiménez and Ferre, 2011).

Pediatric obesity is closely related to dyslipidemias and has been associated with increased risk of cardiovascular diseases, insulin resistance, hypertension, accelerated atherosclerosis (Klop et al., 2013; McGill et al., 2002), metabolic syndrome, reproductive and sleepiness disorders and psychological alterations (Seth and Sharma, 2013).

Genetic factors are the main cause of pediatric dyslipidemias; however, environmental factors such as diet and a lack of physical exercise may contribute to inducing changes in the lipid profile (Ross, 2016). Statins are first-line therapy medications recommended for children with dyslipidemia in order to improve their lipid profile by diminishing LDL-cholesterol, VLDL-cholesterol and triglycerides, as well as increasing HDL-cholesterol, when such lifestyle changes as adherence to an appropriate diet and regular practice of physical exercise are not sufficient to diminish serum total cholesterol (Jiménez and Ferre, 2011; Ross, 2016).

Statins are inhibitors of the enzyme 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which is a limiting enzyme to the biosynthesis of the cholesterol molecule (Istvan and Deisenhofer, 2001; Jiménez and Ferre, 2011); thus, statins are capable of diminishing total cholesterol, decreasing mainly LDL-cholesterol (Istvan and Deisenhofer, 2001; Tandon et al., 2005; Endres, 2006).

These drugs play relevant roles in the organism through their pleiotropic effects that are related to the diminution of intermediate isoprenoids such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Adam and Laufs, 2008). The main pleiotropic effects include atherosclerosis stabilization, improvement on endothelial function and higher nitric oxide

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http://dx.doi.org/10.1016/j.fct.2017.09.003

**Vitamin C partially prevents reproductive damage in adult male rats exposed to rosuvastatin during prepuberty**

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**Abstract**

Pediatric obesity is closely associated with dyslipidemias and environmental factors, such as diet and lack of physical exercises, which may alter lipid profile in children. Rosuvastatin decreases serum total cholesterol and triglycerides concentrations. Vitamin C (ascorbic acid) plays an important role on sperm integrity and fertility. Juvenile male rats were distributed into six experimental groups that received saline solution 0.9%, 3 or 10 mg/Kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 3 or 10 mg/Kg/day of rosuvastatin co-administered with 150 mg/day of ascorbic acid from post-natal (PND) 23 until PND 53 and then the rats were maintained until sexual maturity. Rosuvastatin-exposed groups showed lower sperm quality, androgen depletion and germ cell death. Ascorbic acid was capable to prevent partially the reproductive adverse effects provoked by rosuvastatin. In conclusion, prepubertal exposure to rosuvastatin provokes long-term reproductive damages at sexual maturity and ascorbic acid supplementation at prepuberty may be a preventive mode against these reproductive adverse effects.

**Key words:** Ascorbic acid; rosuvastatin; male reproduction; toxicology.

## 1. Introduction

Obesity, one of the greatest emerging problems of public health, has been affecting adults, children and adolescents (Jiménez & Ferre, 2011). Moreover, obesity is not only a common metabolic and nutritional disorder in developed countries but is also affecting underdeveloped countries (Jiménez & Ferre, 2011).

Pediatric obesity is closely related to dyslipidemias and has been associated with increased risk of cardiovascular diseases, insulin resistance, hypertension, accelerated atherosclerosis (Klop et al., 2013; McGill et al., 2002), metabolic syndrome, reproductive and sleepiness disorders and psychological alterations (Seth & Sharma, 2013).

Genetic factors are the main cause of pediatric dyslipidemias; however, environmental factors such as diet and a lack of physical exercise may contribute to inducing changes in the lipid profile (Ross, 2016). Statins are first-line therapy medications recommended for children with dyslipidemia in order to improve their lipid profile by diminishing LDL-cholesterol, VLDL-cholesterol and triglycerides, as well as increasing HDL-cholesterol, when such lifestyle changes as adherence to an appropriate diet and regular practice of physical exercise are not sufficient to diminish serum total cholesterol (Jiménez & Ferre, 2011; Ross, 2016).

Statins are inhibitors of the enzyme 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which is a limiting enzyme to the biosynthesis of the cholesterol molecule (Istvan & Deisenhofer, 2001; Jiménez & Ferre, 2011); thus, statins are capable of diminishing total cholesterol, decreasing mainly LDL-cholesterol (Istvan & Deisenhofer, 2001; Tandon *et al.*, 2005; Endres, 2006).

These drugs play relevant roles in the organism through their pleiotropic effects that are related to the diminution of intermediate isoprenoids such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Adam & Laufs, 2008). The main pleiotropic effects include atherosclerosis stabilization, improvement on endothelial function and higher nitric oxide bioavailability, by which they act to prevent cardiovascular diseases (Ludman et al., 2009; Tandon et al., 2005).

Rosuvastatin has pharmacological advantages in relation to the other statins and presents unique characteristic binding with the enzyme HMG-CoA reductase, as well as superior inhibitory effects when compared to the other statins, thus leading to higher decreased levels of total cholesterol (Holdgate et al., 2003; McTaggart, 2003).

Children and adolescents are often exposed to chemical compounds that may modify not only puberty timing but also the acquisition and development of reproductive capability (Stoker et al., 2000). Several studies have reported that reproductive disorders during puberty may affect reproduction in adulthood (Perobelli *et al.*, 2012, 2013; Mantovani **&** Fucic, 2014).

Recent studies have attempted to reverse or diminish the adverse effects provoked by various pathologies, metabolic dysfunctions or even chemical compounds used as relevant medications in several treatments (Fernandes et al., 2011a, 2011b; Mukhopadhyay et al., 2013).

The co-administration of substances that have an antioxidant potential or another mode of action that may prevent or reverse the adverse effects of a chemical compound has been widely studied (Corsetti et al., 2011; Mukhopadhyay et al., 2013; Pandir et al., 2014; Sooriyaarachchi et al., 2012), because many medications are needed for the treatment of metabolic dysfunctions and other diseases (Pandir et al., 2014; Sooriyaarachchi et al., 2012).

Vitamin C, also denominated ascorbic acid, is a water-soluble molecule and an important vitamin for the functioning of the organism (Sönmez et al., 2005), and is essential for norepinephrine and collagen biosynthesis, besides contributing to α-tocopherol recycling (Fernandes et al., 2011a, 2011b). Furthermore, ascorbic acid is an antioxidant compound that is not synthetized by humans; therefore, it is necessary to acquire the vitamin from the diet (Fernandes et al., 2011a).

Ascorbic acid plays a significant role in sperm integrity and fertility, acting as a protective compound for spermatogenesis and as an antioxidant substance against oxidative stress (Agarwal *et al.*, 2005; Eskenazi *et al.*, 2005; Shrilatha **&** Muralidhara, 2007; Fernandes *et al.*, 2011b). In addition, ascorbic acid may increase testosterone concentrations and is found in the epididymal fluid and semen where it prevents excessive lipid peroxidation (Sönmez et al., 2005).

One previous study showed delayed reproductive development in prepubertal male rats exposed to rosuvastatin, accompanied by delayed puberty installation, increased germ cell death in the testis, delayed epididymal development during the postnatal period, androgen depletion (Leite et al., 2017, 2014) and diminished expression of androgen receptors in both the testes and the epididymis (Leite et al., 2014). A previous *in vitro* study assessing rat Leydig cells treated with statins showed reduced testosterone production when stimulated by LH (Klinefelter et al., 2014). Another study of men observed that rosuvastatin administration decreased testosterone levels, without affecting sexual function (Hsieh and Huang, 2016). In addition, a previous study on another statin, named atorvastatin, showed reduced sperm quality in men treated with the drug during adulthood (Pons-Rejraji et al., 2014). According to these several studies, statins seem able to interfere with male reproduction.

Considering the utilization of lipid-lowering drugs by children and adolescents and the delay in the reproductive development of prepubertal male rats exposed to rosuvastatin, the present study aims to assess the long-term adverse effects of prepubertal exposure to rosuvastatin and whether prepubertal supplementation with ascorbic acid may diminish or prevent the possible reproductive damage in adulthood.

## 2. Material and Methods

### 2.1. Animals

#### 2.1.1. Obtainment of pups and reduction of litters

Male and female (45 days of age) nonpathogenic free Wistar rats were supplied by the Central Biotherium at the State University of São Paulo (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu.

Rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Animals were maintained under controlled conditions for temperature (23 ± 1°C) and lighting (12:12h photoperiod). The health status of the rats was monitored throughout the experiment. Standard rodent chow (Purina  Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During sexual maturity two nulliparous female rats (75 days of age) were mated with one male (90 days of age) during the dark phase of the lighting cycle; the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating female rats were maintained in individual cages.

After birth, the number of pups per litter was reduced to eight on postnatal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not included in the experimental protocol and were euthanized.

**2.1.2. Experimental design**

On PND 23, male pups were distributed into six experimental groups (n=10 per group, with one pup per litter for each group), that received vehicle (saline solution 0.9%, control group), supplementation with 150 mg/day of ascorbic acid (AA), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0.9% (3 mg or 10 mg) or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin (3 mg + AA or 10 mg + AA). Ascorbic acid and rosuvastatin were purchased from Farmácia Botica Oficinal (Botucatu/Brazil). The treatments were administered by gavage from PND 23 until PND 53, following the male pubertal assay of 31 days recommended by the U.S. Environmental Protection Agency (EPA) (Stoker et al., 2000).

The doses of rosuvastatin available to diminish total cholesterol and LDL-cholesterol often used by humans are between 5 and 40 mg/day (Vaughan & Gotto Jr., 2004); thus, the doses used in this study were based on body surface area correction considering the available doses of rosuvastatin for children and their conversion to equivalent doses for juvenile rats (Reagan-Shaw et al., 2008). The doses of ascorbic acid supplementation were based on previous studies (Fernandes et al., 2011a, 2011b). During the treatment, the rats were monitored in relation to indications of distress, such as the presence of bristling hair and the ingestion of food and water. Rats were maintained until sexual maturity, when a sexual behavior test was performed, followed by euthanasia of the rats ten days later.

The experimental protocol followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and was approved by the Biosciences Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

Male rats were euthanized on PND 110, after performing the sexual behavior test on PND 100, in order to evaluate the long-term reproductive effects due to rosuvastatin exposure and/or ascorbic acid supplementation at prepuberty. Therefore, the following parameters were obtained: final body weight, reproductive and vital organ weights, testicular and epididymal histopathology, testicular morphometry, sperm counts, motility and morphology and hormonal serum concentrations. Moreover, the rats were evaluated in relation to their reproductive performance and fertility after the sexual behavior test.

**2.2. Evaluation of male sexual behavior**

Male rats were placed individually in polycarbonate crystal cages, measuring 44 x 31 x 16 cm, five minutes before the introduction of one adult female in natural proestrus or estrus (sexually receptive) determined by vaginal smear. Behavioral testing was conducted in the dark period of the cycle between 8:00 am and 12 pm in a separate room under dim red illumination. If the male rat did not mount within the next 10 minutes, a second try was permitted on the next day.

The following measures were recorded (Ahlenius & Larsson, 1984): mount and intromission latencies, defined as the times from introduction of the female in the cage to the first mount and intromission, respectively; the number of intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to the first ejaculation; latency to the first post-ejaculatory intromission, the time to the first intromission after the first ejaculation; the number of intromissions after the first ejaculation and total number of ejaculations. During the second chance, if the male did not mount in the first 10 minutes following the introduction of one receptive adult female into the box, it was considered sexually inactive.

**2.3. Euthanasia of the rats, body weight and organ weights**

Male rats were weighed on PND 110 and euthanized following narcosis by $CO_2$ asphyxiation and thereafter, blood was collected by inferior vena cava, between 9:00 and 11:30 a.m. Reproductive organs, such as, left testis, epididymis and vas deferens, seminal gland (full and empty, without the coagulating gland) and ventral prostate, from the rats were collected and weighed. Vital organs that perform an important role in toxicological parameters, such as kidneys, adrenal glands, liver, thyroid, pituitary and brain were also obtained and weighed.

**2.4. Serum hormonal concentrations**

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal determination. Testosterone, FSH and LH were determined by the double-antibody radioimmunoassay. LH and FSH used specified kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK) and testosterone concentrations were measured by the kit Testosterone Maia (Biochem Immuno System, Allentown, PA, USA). All samples were measured in the same assay to avoid the inter-assay errors. Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone.

**2.5. Sperm motility**

Sperm motility was evaluated immediately after euthanasia. The right epididymal cauda was collected, sperm were obtained and a sample was diluted in 2 mL of the modified HTF medium (Spectrun 90126), then a 10 μL aliquot was transferred to a Mackler chamber. Under a light microscope (20x magnification), 100 spermatozoa were analyzed and classified as: type A, motile with regular and fast progressive movement; type B, motile with non-progressive movement or type C, immotile. Sperm motility was expressed as the percentage of total sperm (Perobelli et al., 2012).

**2.6. Sperm morphology**

Sperm were extracted from the right epididymal cauda of all rats, diluted in modified HTF medium and a sample was obtained and added to 1.0 mL of saline formol. For the analysis, smears were prepared on histological slides that were left to dry for 90 minutes and observed in a phase-contrast microscope (400X magnification); 200 spermatozoa were analyzed per animal (Seed et al., 1996). Morphological abnormalities were classified into general categories pertaining to head morphology (without curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken, isolated, i.e., no head attached, or rolled into a spiral). In addition, the presence and position (proximal, medial or distal) of the cytoplasmic droplet were evaluated in the same sperm (Filler, 1993).

**2.7. Sperm counts in the testis and epididymis, and sperm transit time**

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as described previously (Robb et al., 1978), with the following adaptations: the right testis, decapsulated and weighed soon after collection, was homogenized in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), where mature spermatids were enumerated. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

To obtain the number of mature spermatids per gram of testis and the relative DSP, the number of mature spermatids and the DSP were divided by the weight of the testicular parenchyma. In the same manner, caput/corpus and cauda epididymis parts were cut into small fragments with scissors and homogenized, and sperm enumerated as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each part of the organ by the DSP.

**2.8. Sperm DNA fragmentation (Comet assay)**

The comet assay was used for detecting primary DNA damage (single- and double-strand breaks and alkali-labile sites) in sperm isolated from the cauda epididymis that were maintained in HTF medium stored at -80°C. The alkaline version of the assay was performed according to a previous study (Tice et al., 2000). Briefly, 5 µL of the HTF medium with sperm sample was mixed with 75 µL of low-melting-point agarose (0.5%) and then placed onto slides that had previously been covered with a thin layer of normal-melting-point agarose (1.5 %). The slides were covered with lysis solution (100mM $Na_2$-EDTA, 10mM TrisHCl, 2.5M NaCl, pH 11, containing 40mM DTT and 2% Triton X-100) and incubated for 1 hour at 4 °C. Next, the slides were submitted to a second lysis solution containing proteinase K (100 µg/ml) for 2 hours and 30 min at 37 °C and were subsequently incubated in a horizontal electrophoresis tank containing freshly prepared cold alkaline electrophoresis buffer (300mM NaOH, 1mM Na2 EDTA, pH > 13) for 45 min to allow the DNA to unwind and for alkali-labile site expression in a cold room. Electrophoresis was conducted in the same alkaline buffer for 20 min at 3 V/cm and 270 mA at 4 °C. Then, the slides were washed in PBS solution, rinsed in water, fixed in absolute ethanol and stored at room temperature until analysis. The slides were stained with SYBR® Gold (1:10,000; Invitrogen; Grand Island, NY, USA) immediately before analysis. A total of 100 randomly selected nucleoids per animal was analyzed under 400× magnification with a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill, UK). Tail intensity (% DNA in tail) was used to estimate DNA damage. The slides were prepared in duplicate and all steps were conducted in the dark to prevent additional DNA damage.

## 2.9. Histological procedures

The left testis and epididymis were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 µm cuts (transversal sections of testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE) to evaluate testicular and epididymal morphology under light microscopy. The evaluation was performed in a blind assay and the figures were obtained using a Leica light microscope coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows.

Seminiferous tubule cross-sections were randomly chosen in three non-serial testicular sections per animal obtained with a distance of 50 µm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium).

Histopathological analysis of interstitial tissue and peritubular myoid cells was qualitative; the interstitial analysis aimed to assess Leydig cell morphology and the appearance of blood vessels. Epididymal histopathological analysis was also qualitative to evaluate each region of the organ according to the epithelium, lumen and interstitial tissue morphological appearance.

## 2.10. Testicular morphometric analysis

Tubular and luminal area of the seminiferous tubules and epithelial height of the seminiferous epithelium were measured using a Leica light microscope coupled to a digital camera and a personal computer with the software Leica Qwin Version 3 for Windows. For this, 30 random seminiferous tubules from testicular cross-sections (stage IX of the seminiferous epithelium cycle) per animal (n = 9 or 10 animals⁄ group) were examined blindly at x200 magnification. To assess spermatogenesis kinetics, one hundred random tubular sections per animal (n = 9 or 10 animals⁄ group) in three non-consecutive testis cross-sections were classified into four categories: stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelium cycle (Leblond **&** Clermont, 1952), under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at x200 magnification.

**2.11. Fertility and reproductive performance**

This analysis was carried out following natural mating. In the case of rats that had ejaculated during the evaluation of sexual behavior, couples stayed together for an additional four hours, allowing a greater number of ejaculations. Animals that had been deemed inactive were tested again daily for the next 5 consecutive days during which different receptive females were placed in their boxes during the dark period of the cycle.

Every morning, males were separated from the females, and vaginal smears of each female were examined for the presence of sperm. The day on which sperm were found in the vaginal smear was considered day 0 of gestation (GD 0). On the 20th day of gestation (GD 20) females were weighed and then euthanized following narcosis by $CO_2$ asphyxiation and decapitation. After collection of the uterus and ovaries, the gravid uterus weight was obtained; corpora lutea, implantation sites, reabsorptions and live fetuses were enumerated and fetal and placental weights were determined.

Based on these results, the following were determined: gestation rate = number of pregnant females/ number of inseminated females x 100; fertility potential (efficiency of implantation) = implantation sites/ corpora lutea x 100; rate of post-implantation loss = number of implantations - number of live fetuses/ number of implantations x 100; sex ratio = number of male fetuses/ number of female fetuses x 100.

**2.12. Statistical analysis**

For comparison of the results among the experimental groups, two-way ANOVA was performed, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed on GraphPad Prism (version 5.00).

## 3. Results

**3.1. Hormonal measurements, sexual behavior and reproductive performance**

Serum testosterone concentrations were decreased during adulthood in the rosuvastatin-exposed groups in a dose-dependent manner, when compared to the control group ($p < 0.05$) (Fig. 1). Prepubertal supplementation with ascorbic acid was capable of preventing androgen depletion during sexual maturity (Fig. 1). LH and FSH concentrations were similar among the experimental groups (Fig. 1).

The sexual behavior test did not show significant differences among the experimental groups for assessed parameters that include latency to the first mount, intromission, ejaculation and first post-ejaculatory intromission, as well as the numbers of mounts, intromissions and ejaculations (Table 1).

Reproductive performance and fertility evaluated by natural mating showed an elevated rate of post-implantation loss in the group exposed to the higher dose of rosuvastatin during prepuberty, in comparison with control group ($p < 0.05$) (Table. 2). Ascorbic acid supplementation partially prevented the increased rate of post-implantation loss in the group co-exposed to 10 mg of rosuvastatin and ascorbic acid (Table. 2). The remaining parameters evaluated for reproductive performance and fertility showed no significant differences among the groups (Table 2).

**3.2. Sperm parameters**

Daily sperm production and mature spermatids number per testis, relative daily sperm production and mature spermatids number per gram of organ ($p < 0.05$) were lower in the rosuvastatin-treated groups when compared to the control group (Table 3). Ascorbic acid supplementation at prepuberty was able to prevent the reduction of testicular sperm counts in the co-exposed groups (Table 3).

Sperm counts in the caput and corpus epididymis were lower in the groups treated with rosuvastatin at prepuberty, in comparison with the control group ($p < 0.05$) (Table 3). Ascorbic acid partially prevented the diminished sperm counts in the group co-exposed to 10 mg of the statin and ascorbic acid (Table 3). Rosuvastatin-exposed rats presented a lower number of sperm per gram of caput and corpus and diminished sperm storage in the cauda ($p < 0.05$); however, ascorbic acid supplementation was sufficient to prevent this adverse result (Table 3).

The group treated with the higher dose of the statin exhibited increased sperm transit time in the cauda, in comparison with the control group, although total sperm transit times through the epididymis were similar among the experimental groups (Table 3).

Rosuvastatin-treated groups showed a reduction in the rate of progressive sperm, followed by an increased frequency of non-progressive sperm and immotile sperm, when compared to the control group ($p < 0.05$) (Fig. 2). Ascorbic acid supplementation prevented the decreased frequency of non-progressive sperm and partially avoided the diminution of the progressive sperm percentage and the increased rate of immotile sperm (Fig. 2).

During sexual maturity, the percentage of normally shaped sperm was lower in the groups exposed to rosuvatatin at prepuberty, when compared with the control group ($p < 0.05$) (Table 4). Sperm head abnormalities were increased in the group treated with the lower dose of rosuvastatin whereas the group exposed to the higher dose showed an intermediate rate of sperm head abnormalities between the control group and the group treated with 3 mg of rosuvastatin (Table 4). The main head sperm abnormality in these groups was an isolated sperm head. The sperm tail abnormalities were elevated in the statin-treated groups in a dose-dependent manner while the main abnormality was a broken sperm tail ($p < 0.05$) (Table 4). The percentages of sperm with the cytoplasmic droplet were similar among the groups. Ascorbic-acid-supplemented groups were similar to the control group and showed no augmentation in the rate of sperm abnormalities (Table 4).

Sperm DNA damage was higher in the rosuvastatin-exposed groups in comparison to the control group (Fig. 3). Moreover, the co-exposed groups also showed increased DNA damage (Fig. 3).

**3.3. Histopathology and histomorphometry**

Rosuvastatin-exposed groups presented lower frequencies of normal seminiferous tubules, followed by increased rates of acidophilic germ cells in the germinal epithelium, in comparison with the control group ($p < 0.05$) (Fig. 3 and Table 5). Ascorbic acid supplementation at prepuberty restored the percentage of normal seminiferous tubules and prevented the increased frequency of acidophilic germ cells in the epithelium (Fig. 4 and Table 5).

Spermatogenesis kinetics assessed by categories of seminiferous epithelium stages, the height of the seminiferous epithelium and total and luminal area of the seminiferous tubules did not show significant differences among the groups (Table 5).

Epididymal morphology demonstrated leukocyte infiltrates in the initial segment region in the statin-exposed groups (Fig. 5). Moreover, these groups exhibited an apparent hyperplasia of clear cells in the proximal cauda (Fig. 5). Ascorbic acid supplementation was able to avoid these histopathological alterations (Fig. 5).

### 3.4. Body weight and organ weights

Final body weights did not differ significantly among the experimental groups (Table 6). The groups presented similarity in not only reproductive organ weights – namely those of the testis, epididymis, vas deferens, prostate and seminal gland – but also the vital organ weights – that include those of the pituitary, thyroid, liver, adrenal, kidney and brain (Tables 6 and 7).

## 4. Discussion

The experimental design of the present study was based on the fact that obese children use lipid-lowering drugs during childhood and adolescence to decrease cholesterol and prevent cardiovascular diseases (Leite et al., 2014) and, in adulthood, stop taking medications and control their lipid profile according to health habits. Moreover, we mimicked a diet supplemented with ascorbic acid, an antioxidant vitamin, on the attempt to reduce or prevent the adverse reproductive effects caused by rosuvastatin exposure.

Although we have used normal, non-obese juvenile male *Wistar* rats, this study simulates human exposure to rosuvastatin during prepuberty, since atherosclerosis and fatty streaks are detectable during childhood (Ross, 2016; Wiegman et al., 2015) and, in addition, more people are benefitting from the pleiotropic effects of the statins.

Rosuvastatin is a lipid-lowering drug that reduces total cholesterol and triglycerides (Vaughan **&** Gotto Jr, 2004; Penning-Van Beest *et al.*, 2007) and may reduce testosterone concentrations due to the diminution of serum cholesterol levels (Leite *et al.*, 2014; Hsieh **&** Huang, 2016). A previous *in vitro* study evaluating rat Leydig cells treated with statins showed reduced testosterone production when physiologically stimulated by LH (Klinefelter et al., 2014). In the present study, prepubertal exposure to rosuvastatin decreased testosterone concentrations during adulthood in a dose-dependent manner as a long-term adverse effect of the exposure to the statin. Ascorbic acid supplementation at prepuberty prevented the androgen depletion at sexual maturity. FSH and LH concentrations showed no significant differences among the groups.

Statins decrease androgen synthesis by reducing expression and isoprenylation of CYP17A1, as previously demonstrated in an *in vitro* study of theca-interstitial cells (Ortega et al., 2014, 2012). Ascorbic acid is not only responsible for up to 65% of antioxidant action on semen (Fernandes et al., 2011a; Makker et al., 2009), but also for stimulating the steroidogenic enzymes

3β-HSD, 17β-HSD and StAR protein in testes and increasing LH expression in Leydig cells, leading to augmented androgen biosynthesis (Harikrishnan et al., 2013; Radhakrishnakartha et al., 2014).

A recent study has shown that ascorbic acid was able to improve both testosterone concentrations and antioxidant status in pubescent rats exposed to rosuvastatin during prepuberty (Leite et al., 2017), since ascorbic acid is capable of stimulating Leydig-cell steroidogenic enzymes and neutralizing such free radicals as hydrogen peroxide, superoxide and hydroxyl (Harikrishnan et al., 2013; Leite et al., 2017; Makker et al., 2009; Radhakrishnakartha et al., 2014).

Reduced testosterone levels hamper germ-cell survival since Sertoli cells need androgens to bind to their androgen receptors to produce proteins associated with the maintenance and survival of the germ cells (De Gendt et al., 2004; Stanton et al., 2012). Androgen depletion may increase oxidative stress and provoke an augmentation of DNA damage and fragmentation, leading to germ cell death (Stanton et al., 2012). Indeed, an increased amount of sperm DNA damage after exposure to either rosuvastatin dose (3 or 10 mg) was observed. Moreover, juvenile exposure to either rosuvastatin dose increased the percentage of seminiferous tubules with acidophilic germ cells, an indication of germ cell death, probably due to androgen depletion observed during adulthood. Ascorbic acid supplementation was able to prevent the increased rate of germ cell death. However, the height of germinal epithelium, spermatogenesis kinetics and the luminal and total area of the seminiferous tubules were similar among the groups.

The epididymal morphology of rosuvastatin-exposed animals presented augmented inflammation in the initial segment of the organ, which may be related to androgen depletion that increases TNF-α and other inflammatory mediators and leads to inflammation (Chen et al., 2016). Furthermore, epididymis histology of rosuvastatin-treated groups exhibited an apparent hyperplasia at the proximal cauda that seems to be associated with delayed differentiation of epididymal epithelium (Leite et al., 2014).

Diminished sperm counts have been reported as a toxic effect on male reproduction and are correlated with lower fertility in males (Perreault & Cancel, 2001). Abnormalities of sperm head or tail have been utilized as an indicator of damage that is occurring in testes during spermatogenesis due to a toxic action of chemical compounds (Filler, 1993). Moreover, reduced sperm motility is associated with a toxic effect on the epididymis, suggesting adverse effects on sperm maturation (Perreault, 1998; Perreault & Cancel, 2001). In this work, rosuvastatin-treated groups showed reduced sperm motility, diminished sperm counts and increased abnormalities of sperm, thus showing lower sperm quality at sexual maturity. On the other hand, ascorbic acid supplementation might partially prevent these effects, thus improving sperm quality in adulthood.

One previous study showed increased DNA damage in nigrostriatal neurons of mice exposed to different statins, namely pravastatin, simvastatin and atorvastatin (Coetsee et al., 2008). Exposure to toxicants or defective replication during meiosis may produce chromosomal abnormalities such as structural changes or an irregular number of chromosomes, which can result in spontaneous abortions during gestation (Hales et al., 2005). In addition, modifications in paternal DNA sequences or epigenetic changes may interfere with early embryo development (Hales et al., 2005); therefore, the paternal genome integrity is closely related to the embryonic fate (Hales et al., 2005; Marchetti et al., 2003). Rosuvastatin-treated groups exhibited increased DNA fragmentation, but only the group exposed to the highest rosuvastatin dose showed an augmented rate of post-implantation loss after natural mating, indicating that lower integrity of sperm DNA may impair embryo development. Ascorbic acid supplementation partially diminished the rate of post-implantation loss in the co-exposed group. On the other hand, sexual behavior and other parameters employed to assess reproductive performance did not show significant differences among the experimental groups.

The assessment of final body weight supplies relevant information about the health of the rats (Clegg et al., 2001). In association with body weight, reproductive organ weights provide information about the reproductive status (Clegg et al., 2001), whereas vital organ weights indicate the health condition of the respective organs. In this study, final body weight and the reproductive and vital organ weights were found to be similar among the experimental groups.

In summary, prepubertal exposure to rosuvastatin increased the frequency of germ cell death in the testes, provoked epididymal inflammation, hyperplasia of clear cells in the epididymis cauda and reduced sperm quality, at both doses. Furthermore, exposure to the higher dose of the statin increased the rate of post-implantation loss. Ascorbic acid supplementation during prepuberty partially avoided the reproductive impairment at sexual maturity. Reproductive damage provoked by juvenile exposure to rosuvastatin and the main protective effects of ascorbic acid during prepuberty (Leite et al., 2017) appeared to persist into sexual maturity.

## 5. Conclusions

Prepubertal exposure to rosuvastatin diminishes testosterone concentration and sperm quality, increases sperm DNA fragmentation and impairs testicular and epididymal structure during adulthood, in rats. On the other hand, ascorbic acid supplementation partially ameliorates the rosuvastatin-induced damage. We conclude that juvenile exposure to rosuvastatin is a probable risk for male reproduction, whereas ascorbic acid supplementation during the same period may be a means of preventing, at least partially, the long-term reproductive damage generated by rosuvastatin therapy.

**Declaration of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgments**

The authors are grateful to the São Paulo Research Foundation (FAPESP) (Grants #013/22495-1 and #014/13659-3), National Council for Scientific and Technological Development (CNPq) (Grant 308842/2013-8) and Coordination for the Improvement of Higher Education Personnel (CAPES) for their financial support, to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for his excellent technical assistance and to Dr. Daisy Maria Fávero Salvadori, from the Department of Pathology, Medical School, UNESP, Botucatu/SP – Brazil, for her relevant contributions with the Sperm Comet Assay.

**References**

Adam, O., Laufs, U., 2008. Antioxidative effects of statins. Arch. Toxicol. doi:10.1007/s00204-008-0344-4

Agarwal, A., Prabhakaran, S.A., Said, T.M., 2005. Prevention of Oxidative Stress Injury to Sperm. J. Androl. 26, 654–660. doi:10.2164/jandrol.05016

Ahlenius, S., Larsson, K., 1984. Apomorphine and Haloperidol-Induced Effects on Male-Rat Sexual-Behavior - No Evidence for Actions Due to Stimulation of Central Dopamine Autoreceptors. Pharmacol. Biochem. Behav. 21, 463–466.

Chen, C.-W., Jian, C.-Y., Lin, P.-H., Chen, C.-C., Lieu, F.-K., Soong, C., Hsieh, C.-C., Wan, C.-Y., Idova, G., Hu, S., Wang, S.-W., Wang, P.S., 2016. Role of testosterone in regulating induction of TNF-α in rat spleen via ERK signaling pathway. Steroids 111, 148–154. doi:10.1016/j.steroids.2016.03.007

Clegg, E.D., Perreault, S.D., Klinefelter, G.R., 2001. Assessment of male reproductive toxicology, in: Hayes, A.. (Ed.), Principles and Methods of Toxicology. Taylor & Francis, Philadelphia., pp. 1263–1299.

Coetsee, T.N., Pretorius, P.J., Terre'Blanche, G., Bergh, J.J., 2008. Investigating the potential neuroprotective effects of statins on DNA damage in mouse striatum. Food Chem. Toxicol. 46, 3186–3192. doi:10.1016/j.fct.2008.07.006

Corsetti, G., Stacchiotti, A., Tedesco, L., D'Antona, G., Pasini, E., Dioguardi, F.S., Nisoli, E., Rezzani, R., 2011. Essential amino acid supplementation decreases liver damage induced by chronic ethanol consumption in rats. Int. J. Immunopathol. Pharmacol. 24, 611–619.

De Gendt, K., Swinnen, J. V, Saunders, P.T.K., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M., Verhoeven, G., 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. U. S. A. 101, 1327–32. doi:10.1073/pnas.0308114100

Endres, M., 2006. Statins: Potential new indications in inflammatory conditions. Atheroscler. Suppl. doi:10.1016/j.atherosclerosissup.2006.01.005

Eskenazi, B., Kidd, S.A., Marks, A.R., Sloter, E., Block, G., Wyrobek, A.J., 2005. Antioxidant intake is associated with semen quality in healthy men. Hum. Reprod. 20, 1006–1012. doi:10.1093/humrep/deh725

Fernandes, G.S.A., Fernandez, C.D.B., Campos, K.E., Damasceno, D.C., Anselmo-Franci, J.A., Kempinas, W.D.G., 2011a. Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. Reprod. Biol. Endocrinol. 100, 1–9. doi:10.1186/1477-7827-9-100

Fernandes, G.S.A., Gerardin, D.C.C., Assumpção, T.A., Campos, K.E., Damasceno, D.C., Pereira, O.C.M., Kempinas, W.D.G., 2011b. Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats? Pharmacol. Reports 63, 983–991.

Filler, R., 1993. Methods for evaluation of rats epididymal sperm morphology, in: Chapin, R.E., Heindel, J.H. (Eds.), Male Reproductive Toxicology. Press, Academic, San Diego, pp. 334–343.

Hales, B.F., Aguilar-Mahecha, A., Robaire, B., 2005. The stress response in gametes and embryos after paternal chemical exposures. Toxicol. Appl. Pharmacol. 207, 514–520. doi:10.1016/j.taap.2004.12.021

Harikrishnan, R., Abhilash, P.A., Syam Das, S., Prathibha, P., Rejitha, S., John, F., Kavitha, S., Indira, M., 2013. Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male guinea pigs. Br. J. Nutr. 110, 689–98. doi:10.1017/S0007114512005739

Holdgate, G.A., Ward, W.H.J., McTaggart, F., 2003. Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. Biochem. Soc. Trans. 31, 528–531. doi:10.1042/BST0310528

Hsieh, C.J., Huang, B., 2016. Rosuvastatin decreases testosterone levels but not sexual function in men with type 2 diabetes. Diabetes Res. Clin. Pract. 120, 81–88. doi:10.1016/j.diabres.2016.07.026

Istvan, E.S., Deisenhofer, J., 2001. Structural mechanism for statin inhibition of HMG-CoA reductase. Science (80-. ). 292, 1160–1164. doi:10.1126/science.1059344

Jiménez, E.G., Ferre, J.A., 2011. Estatinas : Características y Efectos sobre el Control Lipídico en el Niño y Adolescente Obeso. Rev. Clínica Med. Fam. 4, 69–75.

Klinefelter, G.R., Laskey, J.W., Amann, R.P., 2014. Statin drugs markedly inhibit testosterone production by rat Leydig cells in vitro: Implications for men. Reprod. Toxicol. 45, 52–58. doi:10.1016/j.reprotox.2013.12.010

Klop, B., Elte, J.W.F., Cabezas, M.C., 2013. Dyslipidemia in Obesity: Mechanisms and Potential Targets. Nutrients. doi:10.3390/nu5041218

Leblond, C.P., Clermont, Y., 1952. Spermiogenesis of rat, mouse,hamster and guinea pig as revealed by the “periodic acid-fuchsin sulfurous acid” technique. Am. J. Anat. 90, 167–215.

Leite, G.A.A., Figueiredo, T.M., Sanabria, M., Dias, A.F.M.G., Silva, P.V., Martins Junior, A. da C., Barbosa Junior, F., Kempinas, W.D.G., 2017. Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty. Reprod. Toxicol. doi:10.1016/j.reprotox.2017.07.006

Leite, G.A.A., Rosa, J. de L., Sanabria, M., Cavariani, M.M., Franci, J.A.A., Pinheiro, P.F.F., Kempinas, W.D.G., 2014. Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty. Reprod. Toxicol. 44, 93–103. doi:10.1016/j.reprotox.2014.01.004

Ludman, A., Venugopal, V., Yellon, D.M., Hausenloy, D.J., 2009. Statins and cardioprotection - More than just lipid lowering? Pharmacol. Ther. 122, 30–43. doi:10.1016/j.pharmthera.2009.01.002

Makker, K., Agarwal, A., Sharma, R., 2009. Oxidative stress & male infertility. Indian J. Med. Res. 357–367.

Mantovani, A., Fucic, A., 2014. Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. Reprod. Toxicol. doi:10.1016/j.reprotox.2013.06.002

Marchetti, F., Bishop, J.B., Consentino, L., Moore II, L., Wyrobek, A.J., 2003. Paternally Transmitted Chromosomal Aberrations in Mouse Zygotes Determine Their Embryonic Fate. Biol. Reprod. 70, 616–624. doi:10.1095/biolreprod.103.023044

McGill, H.C., McMahan, C.A., Herderick, E.E., Zieske, A.W., Malcom, G.T., Tracy, R.E., Strong, J.P., 2002. Obesity accelerates the progression of coronary atherosclerosis in young men. Circulation 105, 2712–2718. doi:10.1161/01.CIR.0000018121.67607.CE

McTaggart, F., 2003. Comparative pharmacology of rosuvastatin. Atheroscler. Suppl. 4, 9–14. doi:10.1016/S1567-5688(03)00004-7

Mukhopadhyay, P.K., Dey, A., Mukherjee, S., Pradhan, N.K., 2013. The effect of coadministration of alpha-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. J. Basic Clin. Physiol. Pharmacol. 24, 245–253. doi:10.1515/jbcpp-2012-0039

Ortega, I., Cress, A.B., Wong, D.H., Villanueva, J. a, Sokalska, A., Moeller, B.C., Stanley, S.D., Duleba, A.J., 2012. Simvastatin reduces steroidogenesis by inhibiting Cyp17a1 gene expression in rat ovarian theca-interstitial cells. Biol. Reprod. 86, 1–9. doi:10.1095/biolreprod.111.094714

Ortega, I., Villanueva, J.A., Wong, D.H., Cress, A.B., Sokalska, A., Stanley, S.D., Duleba, A.J., 2014. Resveratrol potentiates effects of simvastatin on inhibition of rat ovarian theca-interstitial cells steroidogenesis. J. Ovarian Res. 7, 21. doi:10.1186/1757-2215-7-21

Pandir, D., Kara, O., Kara, M., 2014. Protective effect of bilberry (Vaccinium myrtillus L.) on cisplatin induced ovarian damage in rat. Cytotechnology 66, 677–685. doi:10.1007/s10616-013-9621-z

Penning-Van Beest, F.J.A., Termorshuizen, F., Goettsch, W.G., Klungel, O.H., Kastelein, J.J.P., Herings, R.M.C., 2007. Adherence to evidence-based statin guidelines reduces the risk of hospitalizations for acute myocardial infarction by 40%: A cohort study. Eur. Heart J. 28, 154–159. doi:10.1093/eurheartj/ehl391

Perobelli, J.E., Alves, T.R., de Toledo, F.C., Fernandez, C.D.B., Anselmo-Franci, J.A., Klinefelter, G.R., Kempinas, W.D.G., 2012. Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. Reprod. Toxicol. 33, 308–315. doi:10.1016/j.reprotox.2011.12.011

Perobelli, J.E., Patrão, M.T.C.C., Fernandez, C.D.B., Sanabria, M., Klinefelter, G.R., Avellar, M.C.W., Kempinas, W.D.G., 2013. Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. Reprod. Toxicol. 38, 65–71. doi:10.1016/j.reprotox.2013.03.004

Perreault, S.D., 1998. Gamete toxicology: the impact of new technologies, in: Dekker, M. (Ed.), Reproductive and Developmental Toxicology. KS. Korach, New York, pp. 635–654.

Perreault, S.D., Cancel, A.M., 2001. Significance of incorporating measures of sperm production and function into rat toxicology studies. Reproduction 121, 207–216. doi:10.1530/reprod/121.2.207

Pons-Rejraji, H., Brugnon, F., Sion, B., Maqdasy, S., Gouby, G., Pereira, B., Marceau, G., Gremeau, A.-S., Drevet, J., Grizard, G., Janny, L., Tauveron, I., 2014. Evaluation of atorvastatin efficacy and toxicity on spermatozoa, accessory glands and gonadal hormones of healthy men: a pilot prospective clinical trial. Reprod. Biol. Endocrinol. 12, 65. doi:10.1186/1477-7827-12-65

Radhakrishnakartha, H., Appu, A.P., Indira, M., 2014. Ascorbic acid supplementation enhances recovery from ethanol induced inhibition of Leydig cell steroidogenesis than abstention in male guinea pigs. Eur. J. Pharmacol. 723, 73–79. doi:10.1016/j.ejphar.2013.12.010

Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J. 22, 659–661. doi:10.1096/fj.07-9574LSF

Robb, G.W., Amann, R.P., Killian, G.J., 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54, 103–107. doi:10.1530/jrf.0.0540103

Ross, J.L., 2016. Statins in the Management of Pediatric Dyslipidemia. J. Pediatr. Nurs. 31, 723–735. doi:10.1016/j.pedn.2016.07.004

Seed, J., Chapin, R.E., Clegg, E.D., Dostal, L.A., Foote, R.H., Hurtt, M.E., Klinefelter, G.R., Makris, S.L., Perreault, S.D., Schrader, S., Seyler, D., Sprando, R., Treinen, K.A., Veeramachaneni, D.N.R., Wise, L.D., 1996. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: A consensus report. Reprod. Toxicol. 10, 237–244. doi:10.1016/0890-6238(96)00028-7

Seth, A., Sharma, R., 2013. Childhood obesity. Indian J Pediatr 80, 309–317. doi:10.1007/s12098-012-0931-5

Shrilatha, B., Muralidhara, 2007. Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. Reprod. Toxicol. 23, 578–587. doi:10.1016/j.reprotox.2007.02.001

Sönmez, M., Turk, G., Yuce, A., 2005. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology 63, 2063–2072. doi:10.1016/j.theriogenology.2004.10.003

Sooriyaarachchi, M., Narendran, A., Gailer, J., 2012. The effect of sodium thiosulfate on the metabolism of cis-platin in human plasma in vitro. Metallomics 4, 960–967. doi:10.1039/c2mt20076g

Stanton, P.G., Sluka, P., Foo, C.F.H., Stephens, A.N., Smith, A.I., Mclachlan, R.I., Donnell, L.O., 2012. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation: Impact on meiotic cells. PLoS One 7, 1–19. doi:10.1371/journal.pone.0041718

Stoker, T.E., Laws, S.C., Guidici, D.L., Cooper, R.L., 2000. The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function. Toxicol. Sci. 58, 50–59. doi:10.1093/toxsci/58.1.50

Tandon, V., Bano, G., Khajuria, V., Parihar, A., Gupta, S., 2005. Pleiotropic effects of statins. Indian J. Pharmacol. 37, 77–85. doi:10.5772/23502

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel / comet assay : Guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Vaughan, C.J., Gotto Jr, A.M., 2004. Update on Statins: 2003. Circulation 110, 886–892. doi:10.1161/01.CIR.0000139312.10076.BA

Wiegman, A., Gidding, S.S., Watts, G.F., Chapman, M.J., Ginsberg, H.N., Cuchel, M., Ose, L., Averna, M., Boileau, C., Borén, J., Bruckert, E., Catapano, A.L., Defesche, J.C., Descamps, O.S., Hegele, R.A., Hovingh, G.K., Humphries, S.E., Kovanen, P.T., Kuivenhoven, J.A., Masana, L., Nordestgaard, B.G., Pajukanta, P., Parhofer, K.G., Raal, F.J., Ray, K.K., Santos, R.D., Stalenhoef, A.F.H., Steinhagen- Thiessen, E., Stroes, E.S., Taskinen, M.R., Tybjealigrg-Hansen, A., Wiklund, O., Catapano, A.L., Defesche, J.C., Descamps, O.S., Gidding, S.S., Ginsberg, H.N., Hegele, R.A., Humphries, S.E., Kovanen, P.T., Nordestgaard, B.G., Parhofer, K.G., Raal, F.J., Ray, K.K., Santos, R.D., Stalenhoef, A.F.H., Steinhagen-Thiessen, E., Stroes, E.S., Watts, G.F., 2015. Familial hypercholesterolaemia in children and adolescents: Gaining decades of life by optimizing detection and treatment. Eur. Heart J. 36, 2425–2437. doi:10.1093/eurheartj/ehv157

**Figure captions**

**Figure 1.** Serum hormonal concentrations (ng/ml) in male rats at 110 days of age. Values expressed as mean ± standard error of mean (SEM). Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 2.** Sperm motility in rats from the experimental groups at 110 days of age. Values expressed as median and interquartile intervals. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 3.** Sperm DNA fragmentation (Comet assay) in rats of the experimental groups observed by percentage of tail intensity. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 4.** Photomicrography of testicular sections from the experimental groups on postnatal day (PND) 110. Observe the presence of acidophilic germ cells (arrows) in the seminiferous epithelium of the rosuvastatin-exposed groups. Hematoxylin and Eosin (HE). Scale bar = 100µm or 50µm.

**Figure 5.** Photomicrography of epididymal sections from the experimental groups on PND 110. Observe the presence of leukocyte infiltrates (asterisks) in the rosuvastatin-exposed groups. Note an apparent hyperplasia of clear cells in the epididymal proximal cauda of rosuvastatin-exposed groups (arrows). Hematoxylin and Eosin (HE). Scale bar = 100µm.

**Table 1.** Sexual behavior test from the experimental groups on post-natal day (PND) 100.

| | Experimental Groups (n= 7) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Latency to the first mount (s) | 188.56 ± 50.73 | 115.50 ± 50.02 | 198.71 ± 63.96 | 133.25 ± 31.82 | 188.33 ± 57.84 | 151.43 ± 43.44 |
| Number of mounts until de first intromission | 4.33 ± 0.80 | 4.56 ± 1.18 | 8.60 ± 2.47 | 7.30 ± 2.20 | 4.20 ± 0.59 | 9.00 ± 2.77 |
| Latency to the first intromission (s) | 245.00 ± 67.58 | 175.89 ± 61.08 | 207.78 ± 59.46 | 147.12 ± 28.86 | 188.71 ± 62.05 | 208.87 ± 82.59 |
| Number of intromissions until the first ejaculation | 15.50 ± 2.64 | 17.89 ± 2.65 | 19.00 ± 3.28 | 23.00 ± 3.09 | 22.33 ± 1.05 | 24.00 ± 3.39 |
| Latency to the first ejaculation (s) | 1093.12 ± 169.01 | 1270.83 ± 194.47 | 890.86 ± 189.00 | 884.87 ± 161.78 | 797.20 ± 134.89 | 1108.17 ± 159.68 |
| Latency to the first post-ejaculatory intromission (s) | 338.62 ± 21.20 | 375.50 ± 26.13 | 318.00 ± 11.47 | 311.71 ± 16.06 | 255.33 ± 8.59 | 287.83 ± 10.19 |
| Number of post-ejaculatory intromission | 9.37 ± 1.67 | 11.00 ± 4.16 | 9.29 ± 0.24 | 11.71 ± 0.94 | 11.67 ± 4.28 | 9.33 ± 2.15 |
| Number of ejaculations | 2.00 ± 0.33 | 1.50 ± 0.34 | 2.71 ± 0.42 | 2.50 ± 0.42 | 3.00 ± 0.36 | 2.17 ± 0.30 |

Values expressed as mean ± standard error of mean (SEM), $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 2.** Reproductive performance and fertility test from the experimental groups on PND110.

| | Experimental Groups (n=9 or 10/group) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Gestational rate (%) | 100% | 88.89% | 81.82% | 100% | 100% | 88.89% |
| [1]Fertility test (%) | 100 (90.83 – 100) $^{a}$ | 93.54 (92.98 – 100) $^{a}$ | 100 (80.95 – 100) $^{a}$ | 100 (91.29 – 100) $^{a}$ | 96.43 (88.05 – 100) $^{a}$ | 100 (94.64 – 100) $^{a}$ |
| [1]Post-implantation loss (%) | 0 (0 – 0) $^{a}$ | 3.33 (0 – 7.20) $^{a}$ | 12.50 (1.92 – 38.64) $^{b}$ | 0 (0 – 4.55) $^{a}$ | 0 (0 – 2.08) $^{a}$ | 12.94 (1.92 – 21.67) $^{ab}$ |
| [1]Sex ratio (M:F) | 0.93 (0.76 – 1.30) $^{a}$ | 1.00 (0.87 – 1.53) $^{a}$ | 1.08 (0.68 – 1.42) $^{a}$ | 1.00 (0.83 – 1.27) $^{a}$ | 0.71 (0.56 – 1.18) $^{a}$ | 0.73 (0.60 – 1.15) $^{a}$ |
| [2]Body weight (GD20) | 372.20 ± 14.86 $^{a}$ | 392.62 ± 12.70 $^{a}$ | 380.44 ± 13.72 $^{a}$ | 361.63 ± 13.14 $^{a}$ | 395.76 ± 9.85 $^{a}$ | 368.80 ± 18.46 $^{a}$ |
| [2]Gravid uterus | 61.24 ± 2.13 $^{a}$ | 63.92 ± 3.65 $^{a}$ | 56.61 ± 2.83 $^{a}$ | 56.09 ± 1.29 $^{a}$ | 62.22 ± 2.22 $^{a}$ | 57.79 ± 2.34 $^{a}$ |
| [2]Male fetus weight | 3.31 ± 0.08 $^{a}$ | 3.12 ± 0.14 $^{a}$ | 3.29 ± 0.15 $^{a}$ | 3.29 ± 0.07 $^{a}$ | 3.26 ± 0.10 $^{a}$ | 3.40 ± 0.10 $^{a}$ |
| [2] Female fetus weight | 3.13 ± 0.10 $^{a}$ | 3.05 ± 0.11 $^{a}$ | 3.12 ± 0.14 $^{a}$ | 3.11 ± 0.09 $^{a}$ | 3.02 ± 0.09 $^{a}$ | 3.25 ± 0.11 $^{a}$ |
| [2]Placenta weight from male fetus | 0.60 ± 0.01 $^{a}$ | 0.56 ± 0.04 $^{a}$ | 0.58 ± 0.02 $^{a}$ | 0.57 ± 0.02 $^{a}$ | 0.58 ± 0.02 $^{a}$ | 0.59 ± 0.03 $^{a}$ |
| [2] Placenta weight from female fetus | 0.57 ± 0.02 $^{a}$ | 0.56 ± 0.04 $^{a}$ | 0.58 ± 0.02 $^{a}$ | 0.56 ± 0.02 $^{a}$ | 0.57 ± 0.03 $^{a}$ | 0.57 ± 0.02 $^{a}$ |

Values expressed as median and interquartile intervals[1] or mean ± SEM[2], $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups. GD = gestational day.

**Table 3.** Sperm counts in the testis and epididymis from the experimental groups on PND 110.

| Sperm counts | Experimental Groups (n = 10) | | | | | |
|---|---|---|---|---|---|---|
| | Control | 3 mg | 10 mg | AA | 3 mg + AA | 10 mg + AA |
| ***Sperm counts in the testis*** | | | | | | |
| Mature spermatid number ($10^6$/testis) | 193.60 ± 3.36 $^a$ | 153.20 ± 4.07 $^b$ | 153.50 ± 4.11$^b$ | 197.20 ± 4.43 $^a$ | 185.70 ± 4.70 $^a$ | 184.00 ± 5.36 $^a$ |
| Mature spermatid number ($10^6$/g testis) | 112.50 ± 1.97 $^a$ | 85.46 ± 2.13 $^b$ | 83.80 ± 1.71 $^b$ | 106.60 ± 2.93 $^a$ | 109.70 ± 3.66 $^a$ | 108.10 ± 1.55$^a$ |
| Daily sperm production ($10^6$/testis/day) | 31.73 ± 0.55 $^a$ | 25.12 ± 0.66 $^b$ | 25.17 ± 0.67 $^b$ | 32.31 ± 0.72 $^a$ | 30.45 ± 0.77 $^a$ | 30.17 ± 0.88 $^a$ |
| Relative sperm production ($10^6$/g testis/day) | 18.45 ± 0.32 $^a$ | 14.11 ± 0.41 $^b$ | 13.74 ± 0.28 $^b$ | 17.47 ± 0.48 $^a$ | 18.06 ± 0.55 $^a$ | 17.72 ± 0.25 $^a$ |
| ***Sperm counts in the epididymis*** | | | | | | |
| Sperm number in the caput/corpus (x$10^6$/organ) | 140.00 ± 4.98 $^a$ | 101.60 ± 6.53 $^b$ | 99.93 ± 5.22 $^b$ | 126.30 ± 5.10 $^a$ | 107.60 ± 4.42 $^b$ | 116.00 ± 6.32 $^{ab}$ |
| Relative sperm number in the caput/corpus (x$10^6$/g/organ) | 469.30 ± 15.11 $^a$ | 372.30 ± 13.69 $^b$ | 355.60 ± 14.58 $^b$ | 463.40 ± 13.03 $^a$ | 458.00 ± 20.91 $^a$ | 421.80 ± 13.68 $^a$ |
| Sperm number in the cauda (x$10^6$/organ) | 238.40 ± 3.80 $^a$ | 203.80 ± 7.11 $^b$ | 207.90 ± 7.31 $^b$ | 238.60 ± 4.94 $^a$ | 232.50 ± 8.96 $^a$ | 225.80 ± 8.89 $^a$ |
| Sperm transit time in the caput/corpus (days) | 4.42 ± 0.16 $^a$ | 4.06 ± 0.25 $^a$ | 4.01 ± 0.25 $^a$ | 3.92 ± 0.18 $^a$ | 3.64 ± 0.18 $^a$ | 3.83 ± 0.14 $^a$ |
| Sperm transit time in the cauda (days) | 7.53 ± 0.16 $^a$ | 8.13 ± 0.22 $^a$ | 8.27 ± 0.22 $^b$ | 7.40 ± 0.12 $^a$ | 7.65 ± 0.27 $^a$ | 7.51 ± 0.26 $^a$ |
| Total sperm transit time (days) | 11.94 ± 0.26 $^a$ | 12.18 ± 0.38 $^a$ | 12.27 ± 0.35 $^a$ | 11.32 ± 0.25 $^a$ | 11.41 ± 0.39 $^a$ | 11.34 ± 0.29 $^a$ |

Values expressed as mean ± SEM, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 4.** Sperm morphology from the experimental groups on PND110.

| Sperm morphology (%) | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Normal shaped sperm | 94.25 (92.28 – 95.00) [a] | 88.75 (87.50 – 90.50) [b] | 90.75 (87.50 – 91.00) [b] | 93.25 (91.88 – 94.38) [a] | 91.25 (88.38 – 93.25) [a] | 92.00 (91.13 – 95.25) [a] |
| Abnormalities of the sperm head | 3.75 (3.00 – 6.25) [a] | 7.00 (5.63 – 11.00) [b] | 6.50 (5.25 – 10.25) [ab] | 4.25 (1.38 – 6.13) [a] | 6.75 (4.25 – 9.00) [a] | 5.50 (1.62 – 7.75) [a] |
| Isolated sperm head | 3.75 (3.00 – 5.50) [a] | 7.00 (5.63 – 10.63) [b] | 6.50 (5.25 – 10.25) [ab] | 4.25 (1.38 – 6.13) [a] | 6.75 (4.25 – 9.00) [a] | 5.50 (1.25 – 7.77) [a] |
| Abnormalities of the sperm tail | 1.75 (1.38 – 2.63) [a] | 3.25 (2.25 – 4.63) [ab] | 4.00 (2.50 – 4.37) [b] | 2.75 (1.00 – 3.63) [a] | 2.00 (1.50 – 2.63) [a] | 2.25 (1.25 – 3.12) [a] |
| Broken sperm tail | 0 (0 – 0) [a] | 0 (0 – 1.00) [a] | 0 (0.50 – 1.13) [b] | 0 (0.63 –1.00) [a] | 0 (0.50 – 0.50) [a] | 0 (0.50 – 0.50) [a] |
| Sperm with cytoplasmic droplet | 7.25 (3.88 – 10.50) [a] | 9.75 (8.38 – 11.88) [a] | 9.75 (7.50 – 11.63) [a] | 7.25 (6.13 – 9.50) [a] | 8.50 (6.88 – 9.63) [a] | 8.00 (7.00 – 9.25) [a] |

Values expressed as median and interquartile intervals, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 5.** Testicular histopathological and morphometric evaluation from the experimental groups on PND 110.

| | Experimental Groups (n=9 or 10/group) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| [1]Normal seminiferous tubules (%) | 96.50 (96.50 – 97.50) [a] | 94.50 (89.50 – 96.00) [b] | 93.00 (92.00 – 94.00) [b] | 97.00 (96.00 – 97.50) [a] | 95.00 (95.00 – 97.00) [a] | 96.00 (94.00 – 97.00) [a] |
| [1]Seminiferous tubules with acidofilic cells (%) | 2.50 (2.00 – 3.00) [a] | 4.00 (3.00 – 5.50) [b] | 6.50 (3.50 – 6.50) [b] | 2.00 (1.50 – 3.50) [a] | 3.50 (2.50 – 3.50) [a] | 3.50 (2.50 – 4.00) [a] |
| [2]Height of the germinal epithelium (μm) | 87.20 ± 1.75 [a] | 87.06 ± 1.25 [a] | 85.28 ± 1.02 [a] | 85.05 ± 0.84 [a] | 86.32 ± 0.89 [a] | 84.64 ± 1.02 [a] |
| [2]Total area of the seminiferous tubule (μm²) | 61,355.47± 1.546.41 [a] | 63,052.42 ± 1774.16 [a] | 61,199.02 ± 2.160.30 [a] | 59,376.42 ± 2.520.93 [a] | 60,739.10 ± 1.396.99 [a] | 60,228.03 ± 1.595.18 [a] |
| [2]Luminal area of the seminiferous tubule (μm²) | 8,690.01 ± 467.45 [a] | 9,367.66 ± 417.30 [a] | 9,309.02 ± 745.01 [a] | 8,711.65 ± 910.96 [a] | 8,779.09 ± 592.42 [a] | 9,103.56 ± 482.98 [a] |
| [1]Stages I – VI (%) | 31.50 (30.50 – 31.50) [a] | 33.50 (30.00 – 35.00) [a] | 31.50 (29.50 – 32.50) [a] | 33.50 (33.00 – 34.50) [a] | 33.00 (30.50 – 37.50) [a] | 31.00 (28.50 – 34.50) [a] |
| [1]Stages VII – VIII (%) | 30.50 (28.50 – 34.50) [a] | 33.50 (32.50 – 34.00) [a] | 32.50 (31.00 – 34.00) [a] | 32.00 (29.00 – 35.00) [a] | 30.00 (26.00 – 33.00) [a] | 31.50 (28.00 – 33.50) [a] |
| [1]Stages IX – XIII (%) | 33.00 (30.50 – 33.50) [a] | 29.50 (27.00 – 32.50) [a] | 32.00 (31.00 – 33.00) [a] | 31.50 (27.50 – 33.00) [a] | 33.00 (28.50 – 34.00) [a] | 35.50 (29.00 – 37.00) [a] |
| [1]Stage XIV (%) | 4.50 (4.00 – 5.50) [a] | 4.50 (3.00 – 6.00) [a] | 3.50 (3.50 – 4.50) [a] | 5.00 (3.00 – 5.50) [a] | 4.50 (2.50 – 5.00) [a] | 3.50 (3.00 – 4.50) [a] |

Values expressed as median and interquartile intervals[1] or mean ± SEM[2], $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 6.** Body weight and reproductive organ weights from the experimental groups on PND 110.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Final body weight (g) | 414.00 ± 9.54 | 426.70 ± 10.00 | 424.60 ± 11.07 | 409.80 ± 10.87 | 411.60 ± 9.59 | 381.20 ± 13.36 |
| Testis (g) | 1.77 ± 0.04 | 1.81 ± 0.04 | 1.88 ± 0.06 | 1.88 ± 0.04 | 1.75 ± 0.04 | 1.75 ± 0.04 |
| Testis (g/100g BW) | 0.43 ± 0.01 | 0.43 ± 0.01 | 0.44 ± 0.02 | 0.46 ± 0.02 | 0.43 ± 0.01 | 0.47 ± 0.02 |
| Epididymis (mg) | 622.20 ± 13.27 | 637.80 ± 12.12 | 638.90 ± 23.79 | 632.50 ± 12.59 | 614.80 ± 10.09 | 619.50 ± 12.44 |
| Epididymis (mg/100g BW) | 150.90 ± 4.48 | 149.90 ± 3.42 | 150.20 ± 8.20 | 155.30 ± 5.21 | 149.80 ± 3.08 | 167.80 ± 4.94 |
| Vas deferens (mg) | 81.66 ± 2.07 | 83.42 ± 3.21 | 82.71 ± 3.54 | 84.73 ± 2.51 | 81.99 ± 2.75 | 79.22 ± 2.13 |
| Vas deferens (mg/100g BW) | 19.79 ± 0.57 | 19.66 ± 0.93 | 19.50 ± 1.27 | 20.78 ± 0.73 | 20.00 ± 0.74 | 21.54 ± 0.92 |
| Prostate (mg) | 483.40 ± 26.74 | 551.20 ± 40.53 | 506.60 ± 35.06 | 451.20 ± 23.56 | 519.10 ± 48.19 | 449.00 ± 35.39 |
| Prostate (mg/100g BW) | 117.10 ± 6.48 | 128.90 ± 8.72 | 119.30 ± 9.95 | 111.70 ± 7.80 | 126.70 ± 12.14 | 120.60 ± 8.28 |
| Full seminal gland (mg) | 1.52 ± 0.08 | 1.63 ± 0.07 | 1.57 ± 0.09 | 1.51 ± 0.07 | 1.58 ± 0.07 | 1.42 ± 0.06 |
| Full seminal gland (mg/100g BW) | 366.10 ± 19.27 | 384.60 ± 19.14 | 369.40 ± 25.19 | 368.80 ± 17.22 | 383.30 ± 14.57 | 381.60 ± 15.99 |
| Empty seminal gland (mg) | 440.20 ± 33.82 | 451.60 ± 31.13 | 495.30 ± 55.42 | 435.80 ± 26.88 | 475.10 ± 26.18 | 457.50 ± 19.72 |
| Empty seminal gland (mg/100g BW) | 106.50 ± 8.32 | 107.90 ± 8.98 | 118.40 ± 16.97 | 106.60 ± 6.41 | 115.80 ± 6.66 | 124.30 ± 7.11 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Table 7.** Vital organ weights from the experimental groups on PND 110.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Pituitary (mg) | 10.04 ± 0.42 | 10.28 ± 0.35 | 9.96 ± 0.47 | 9.84 ± 0.49 | 10.04 ± 0.53 | 9.81 ± 0.47 |
| Pituitary (mg/100g BW) | 2.43 ± 0.09 | 2.41 ± 0.07 | 2.32 ± 0.10 | 2.37 ± 0.14 | 2.47 ± 0.17 | 2.66 ± 0.14 |
| Thyroid (mg) | 18.18 ± 0.98 | 18.40 ± 1.12 | 18.56 ± 1.26 | 19.20 ± 1.09 | 18.54 ± 1.04 | 17.51 ± 0.70 |
| Thyroid (mg/100g BW) | 4.40 ± 0.24 | 4.32 ± 0.26 | 4.34 ± 0.30 | 4.68 ± 0.23 | 4.51 ± 0.24 | 4.74 ± 0.19 |
| Liver (g) | 17.16 ± 0.58 | 18.84 ± 0.75 | 16.97 ± 0.74 | 16.29 ± 0.48 | 16.38 ± 0.47 | 15.27 ± 0.70 |
| Liver (g/100g BW) | 4.14 ± 0.10 | 4.31 ± 0.11 | 3.94 ± 0.11 | 3.98 ± 0.07 | 3.98 ± 0.07 | 4.10 ± 0.09 |
| Adrenal (mg) | 37.89 ± 2.20 | 41.51 ± 0.74 | 34.64 ± 1.68 | 37.00 ± 2.50 | 37.16 ± 2.18 | 34.74 ± 2.58 |
| Adrenal (mg/100g BW) | 9.16 ± 0.50 | 9.78 ± 0.31 | 8.06 ± 0.34 | 9.08 ± 0.66 | 9.07 ± 0.59 | 9.39 ± 0.70 |
| Kidney (g) | 1.51 ± 0.03 | 1.57 ± 0.06 | 1.58 ± 0.05 | 1.48 ± 0.03 | 1.48 ± 0.04 | 1.47 ± 0.03 |
| Kidney (g/100g BW) | 0.37 ± 0.01 | 0.37 ± 0.01 | 0.37 ± 0.01 | 0.36 ± 0.01 | 0.36 ± 0.01 | 0.40 ± 0.01 |
| Brain (g) | 2.07 ± 0.02 | 2.05 ± 0.03 | 2.08 ± 0.03 | 2.06 ± 0.05 | 2.05 ± 0.02 | 1.97 ± 0.03 |
| Brain (g/100g BW) | 0.50 ± 0.01 | 0.48 ± 0.01 | 0.49 ± 0.01 | 0.51 ± 0.01 | 0.50 ± 0.01 | 0.54 ± 0.02 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Figure 1.**

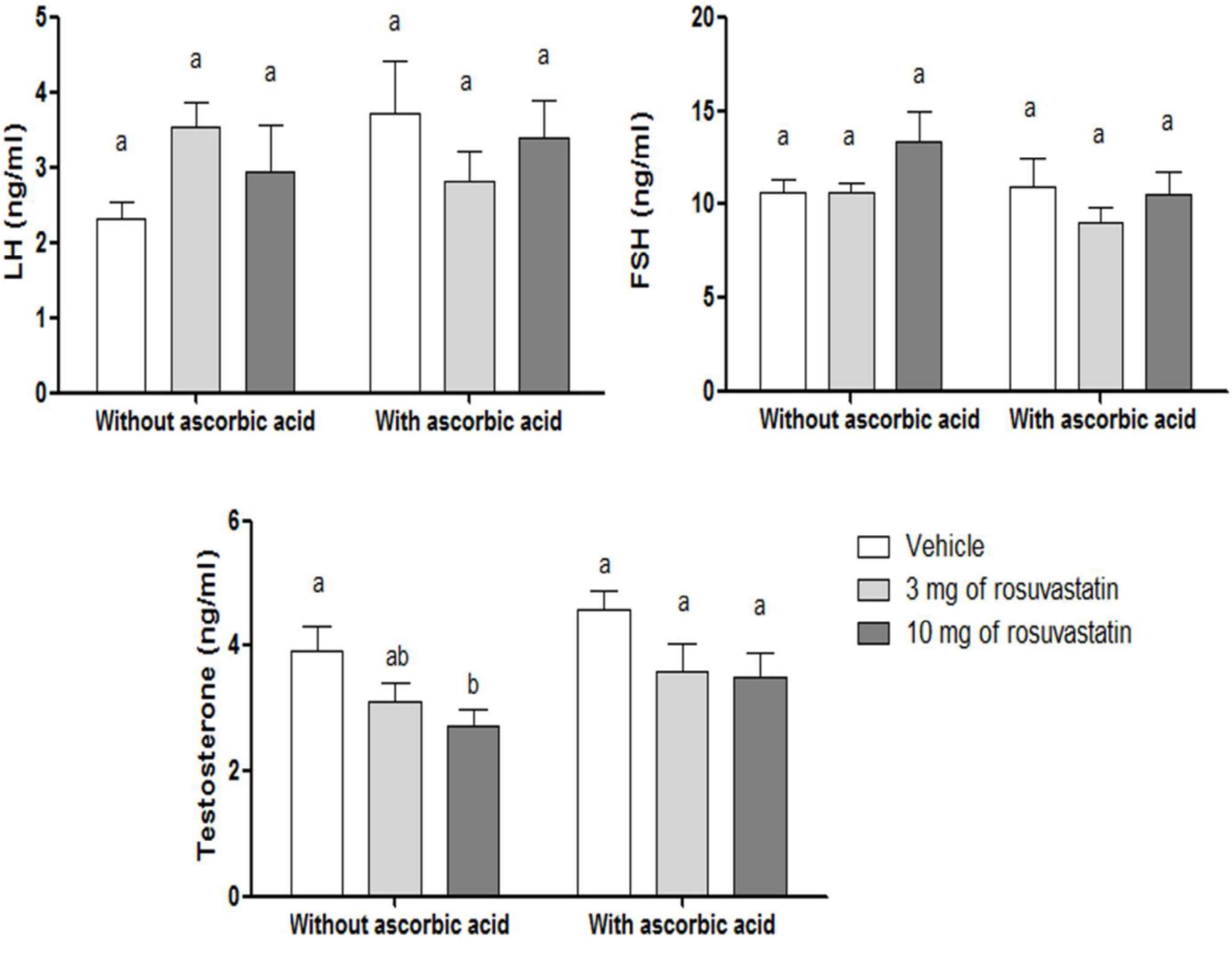

LH (ng/ml)
0
1
2
3
4
5
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
FSH (ng/ml)
0
5
10
15
20
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Testosterone (ng/ml)
0
2
4
6
a
ab
b
a
a
a
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 2.**

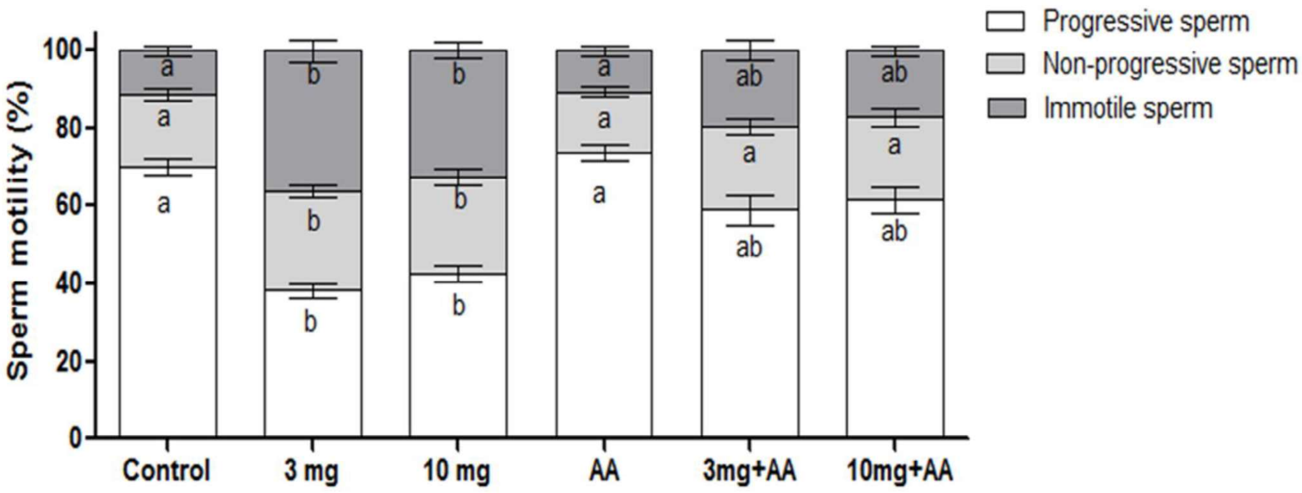

Progressive sperm
Non-progressive sperm
Immotile sperm
Sperm motility (%)
100
80
60
40
20
0
a
a
a
b
b
b
b
b
b
a
a
a
ab
a
ab
ab
a
ab
Control
3 mg
10 mg
AA
3mg+AA
10mg+AA

**Figure 3.**

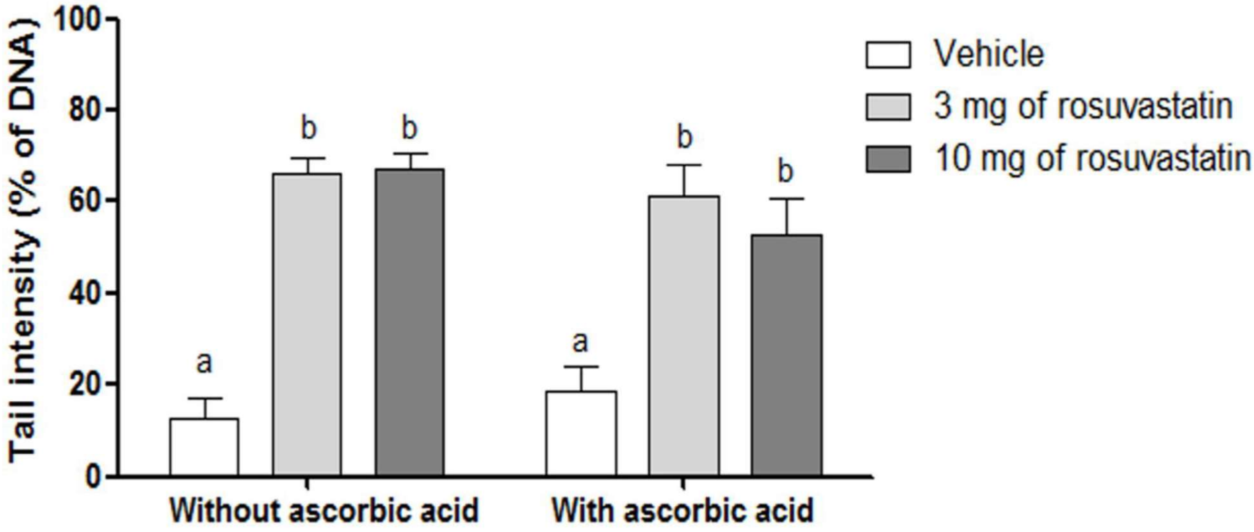

Tail intensity (% of DNA)
100
80
60
40
20
0
a
b
b
a
b
b
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 4.**

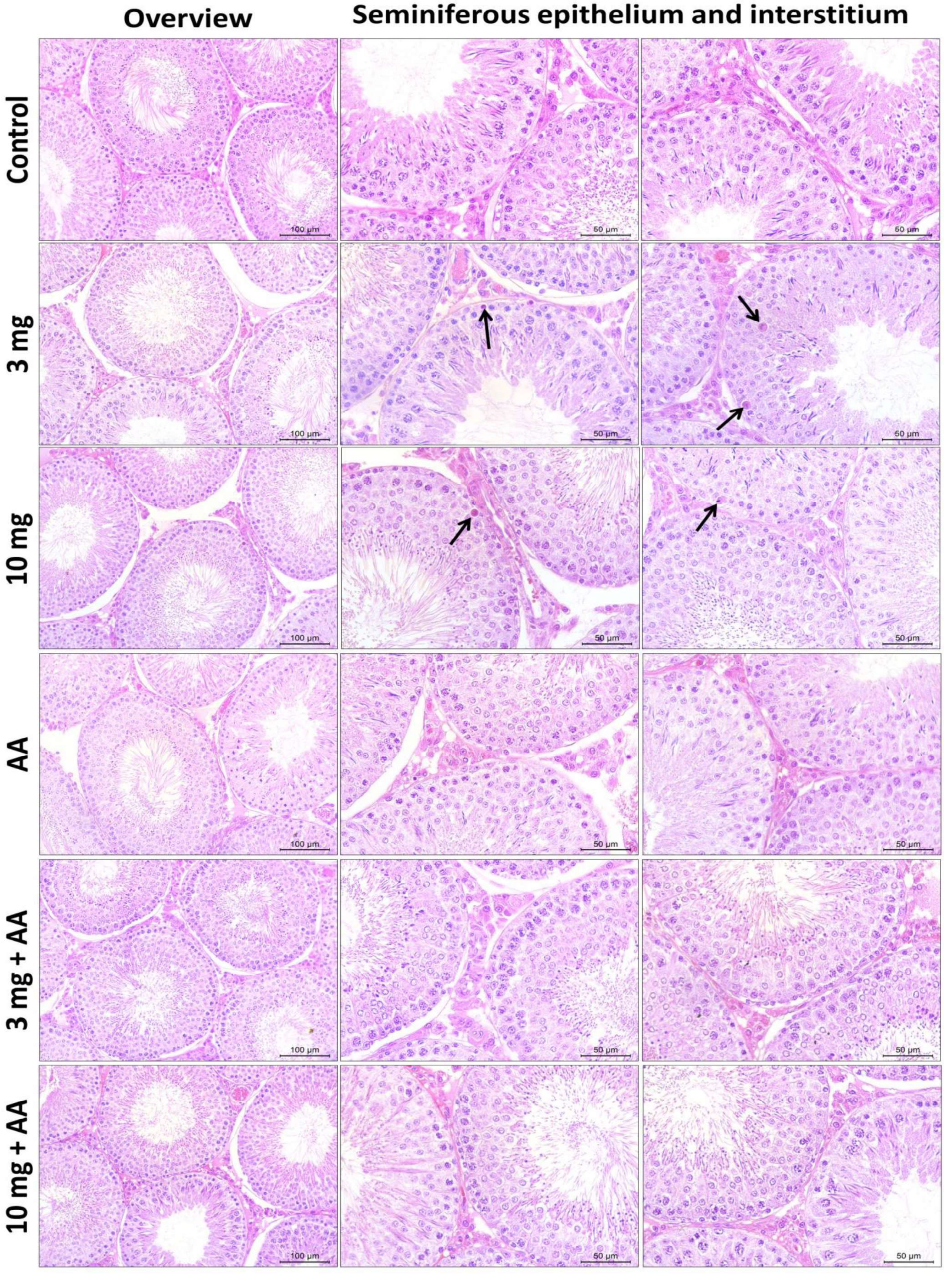
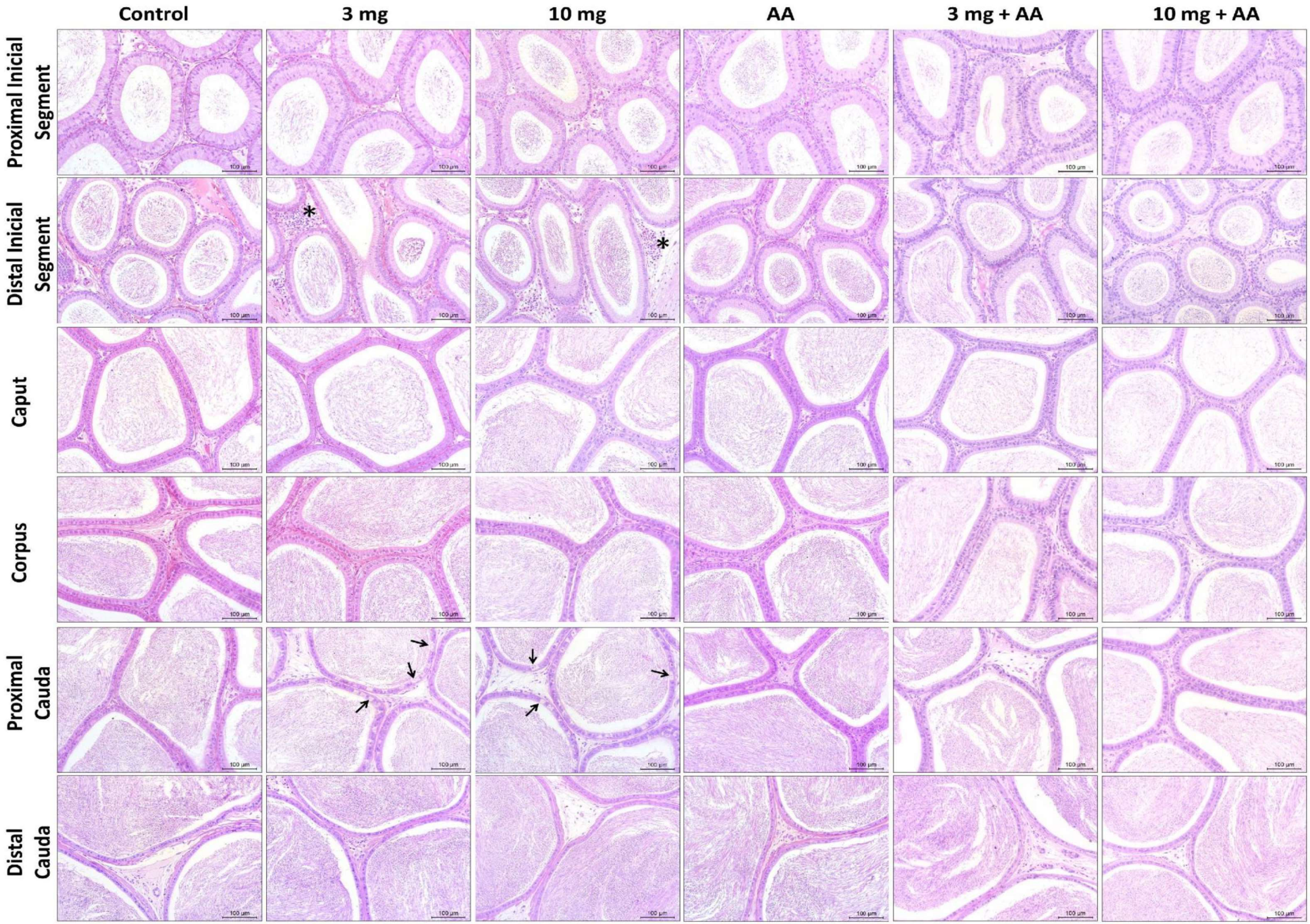

Overview
Seminiferous epithelium and interstitium
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
100 µm
50 µm

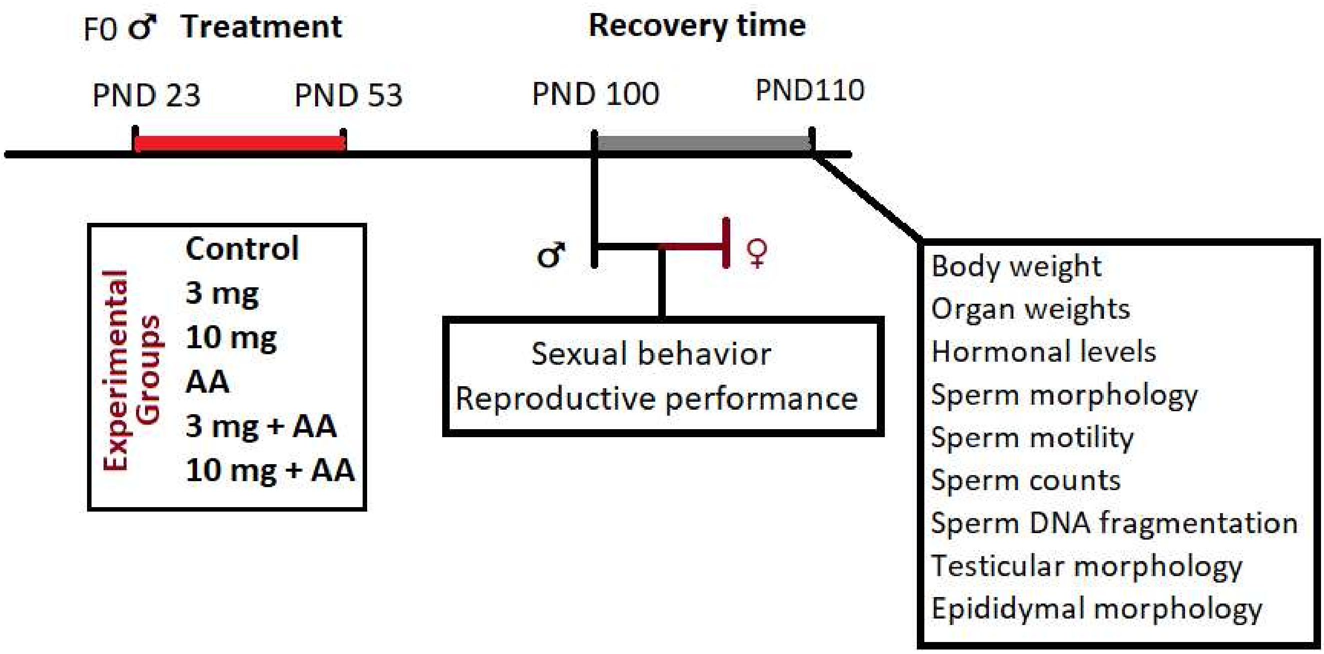
Figure 5.



Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
Proximal Inicial Segment
Distal Inicial Segment
Caput
Corpus
Proximal Cauda
Distal Cauda
100 µm

**Supplementary Material**

**Figure 1.** Description of the experimental design.



# Capítulo 3

**Manuscrito III**

O terceiro manuscrito é intitulado **"Ascorbic acid supplementation ameliorates testicular structural impairment and oxidative stress in male rats exposed to rosuvastatin during pre-puberty"** e será submetido na revista Journal of Applied Toxicology, Wiley, ISSN: 1099-1263. Fator de impacto: 3,159.

**Ascorbic acid supplementation ameliorates testicular structural impairment and oxidative stress in male rats exposed to rosuvastatin during pre-puberty**

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**Short title:** Statin and vitamin C on male rat reproduction

**Keywords:** Statin, Vitamin C, testis, male reproduction, toxicology

**Abstract**

Dyslipidemias are occurring earlier in the population due to the augmentation of obesity. Rosuvastatin reduces cholesterol and triglycerides; however, previous studies have shown that it may affect male reproduction. Ascorbic acid (AA), an antioxidant compound, plays a protective role in the male reproductive system. This study aimed to evaluate whether pre-pubertal exposure to rosuvastatin may impair testicular structure and antioxidant status in male rats and if supplementation with AA may alleviate these damages. Male rats were randomly divided into six experimental groups (n=10), which received saline solution 0.9%, 3 or 10mg/Kg/day of rosuvastatin, 150mg/day of AA or 3 or 10mg/Kg/day of rosuvastatin associated with 150mg/day of AA orally from post-natal day (PND) 23 until PND53. Testicular parameters were assessed on PND53 and 110. There were diminished androgen receptors staining in the Sertoli cells and increased germ cell death in rosuvastatin-exposed groups, in both periods. Spermatids showed lower estrogen alpha-receptors staining in the group exposed to 10mg of statin at adulthood. There were androgen depletion and increased lipid peroxidation and catalase activity in statin-exposed groups. Rosuvastatin exposure during pre-puberty impaired testicular structure, steroid receptor distribution and increased oxidative stress, however, AA was able to ameliorate the impairment provoked by statin exposure.

**Short Abstract**

Juvenile exposure to rosuvastatin impaired testicular structure, androgen and estrogen receptors distribution and increased oxidative stress in the testis. On the other hand, ascorbic acid supplementation during pre-puberty alleviated the damage provoked by rosuvastatin exposure.

## 1. Introduction

Obesity is considered a metabolic and nutritional disorder in developed countries and is also affecting underdeveloped countries nowadays (Jiménez & Ferre, 2011). Furthermore, obesity has been considered as an important emerging problem of public health that affects children, adolescents and adult population (Jiménez & Ferre, 2011).

The frequency of pediatric patients presenting dyslipidemias is augmenting in consequence of the raised prevalence of obesity (Jiménez & Ferre, 2011). Dyslipidemias have been characterized as lipid profile disorders that include augmented serum total cholesterol, LDL-cholesterol and VLDL-cholesterol, increased serum triglycerides or decreased HDL-cholesterol concentrations in the blood (Jiménez & Ferre, 2011; Kwiterovich, 2008a, 2008b). Dyslipidemias in children and adolescents have been associated with genetic factors, such as familial hypercholesterolemia (Cook & Kavey, 2011; Ross, 2016) or related to environmental factors such as inappropriate and sedentary lifestyle, and bad eating habits (Izar, Fonseca, & Fonseca, 2011; Ross, 2016).

Statins are one of the main drug class utilized to improve lipid profile by diminishing total cholesterol (Istvan & Deisenhofer, 2001; Jiménez & Ferre, 2011). They are lipid-lowering drugs that act inhibiting the limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), thus, reducing intermediate isoprenoids and cholesterol, which is the final substance of the pathway (Istvan & Deisenhofer, 2001; Jiménez & Ferre, 2011).

Among statins, rosuvastatin was the last statin launched in the market and is available to be used by population for lipid-lowering purposes (Holdgate, Ward, & McTaggart, 2003; McTaggart, 2003; Olsson, McTaggart, & Raza, 2002). Moreover, it shows superior inhibitory effects on cholesterol biosynthesis in comparison with other statins (Holdgate et al., 2003; McTaggart, 2003; Olsson et al., 2002). Contrary to the benefits, previous studies showed that statins may interfere with male reproductive development (Leite et al., 2014; Leite, Figueiredo, Sanabria, et al., 2017), are able to diminish serum testosterone levels (Hsieh & Huang, 2016; Leite et al., 2014; Leite, Figueiredo, Pacheco, et al., 2017; Leite, Figueiredo, Sanabria, et al., 2017) and decrease sperm quality in adult men treated with the medication (Pons-Rejraji et al., 2014).

The pediatric population is exposed to several chemical compounds and some of them are considered toxicants that may affect puberty timing and development of reproductive system (Stoker, Laws, Guidici, & Cooper, 2000). It is known that changes in puberty timing may interfere with reproduction at sexual maturity, therefore, alterations in this parameter provide relevant information about the reproductive capability at adulthood (Mantovani & Fucic, 2014; Perobelli et al., 2012; Perobelli et al., 2013).

Currently, several studies have been tried to prevent or ameliorate the impairment provoked by metabolic dysfunctions, pathologies or even the adverse effects of chemical substances utilized as important medications in different therapies (Fernandes, Fernandez, et al., 2011; Fernandes, Gerardin, et al., 2011; Mukhopadhyay, Dey, Mukherjee, & Pradhan, 2013).

Ascorbic acid is a needful hydrosoluble vitamin for various biochemical reactions in the organism, presenting a crucial role in collagen biosynthesis and α-tocopherol recycling (Fernandes, Fernandez, et al., 2011; Fernandes, Gerardin, et al., 2011; Sönmez, Turk, & Yuce, 2005). In addition, it acts as an important antioxidant vitamin against oxidative stress in the testis and plays a protecting role for spermatogenesis (Agarwal, Prabhakaran, & Said, 2005; Eskenazi et al., 2005; Fernandes, Fernandez, et al., 2011; Shrilatha & Muralidhara, 2007). It is known that ascorbic acid may improve testosterone concentrations and prevents excessive lipid peroxidation in epididymal fluid and semen (Sönmez et al., 2005).

Considering the use of lipid-lowering medications by children and adolescents and the evidence of reproductive damage provoked by statin exposure, the present study aims to assess the testicular outcomes in pubescent and adult male rats exposed to rosuvastatin during pre-puberty, besides to assess the possible protective role of ascorbic acid on testis against potential damages provoked by rosuvastatin.

## 2. Material and Methods

### 2.1. Animals

#### 2.1.1. Obtainment of pups and reduction of litters

Male and female nonpathogenic free *Wistar* rats were supplied with 45 days of age from Central Biotherium of São Paulo State University (UNESP), Botucatu/SP and allocated in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu.

Rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Animals were maintained under controlled temperature (23 ± 1°C) and lighting conditions (12:12h photoperiod). The health conditions of the animals was monitored along the experiment. Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During adulthood, two nulliparous receptive female rats with 75 days of age were mated with one male rat (90 days of age) during the dark phase of the lighting cycle and the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating females were maintained in individual cages during the experiment.

After birth, the number of pups per litter was culled to eight on post-natal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not considered into the experiment and were euthanized.

**2.1.2. Experimental design**

On PND 23 male pups were distributed into six experimental groups (n=20 per group, with one pup per litter for each group), that received vehicle (saline solution 0.9%, control group), supplementation with 150 mg/day of ascorbic acid (AA), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0.9% (3 mg or 10 mg) or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin (3 mg + AA or 10 mg + AA). Rosuvastatin and ascorbic acid were purchased from a commercial pharmacy (Farmácia Botica Oficinal, Botucatu/Brazil). Ascorbic acid and rosuvastatin were prepared in a dark room. The treatments were performed by gavage since PND 23 until PND 53, following male pubertal assay of 31 days recommended by Environmental Protection Agency (EPA) (Stoker et al., 2000).

The doses of rosuvastatin available to diminish total cholesterol and triglycerides in humans are between 5 and 40 mg/day (Vaughan & Gotto Jr, 2004), thus the doses used in this study were based on body surface area correction from children available doses of rosuvastatin to pre-pubertal male rats equivalent doses (Reagan-Shaw, Nihal, & Ahmad, 2008). The doses of ascorbic acid supplementation were based on previous studies (Fernandes, Fernandez, et al., 2011; Fernandes, Gerardin, et al., 2011; Leite, Figueiredo, Sanabria, et al., 2017). During the whole experiment, the animals were monitored in relation to the indications of distress, such as the presence of bristling hair and the ingestion of food and water. On PND 53, soon after treatment, male rats (n=10/group) were euthanized to obtain the testes, which were used for the different analyses. The remaining rats (n=10/group) were maintained until sexual maturity, when were euthanized on PND 110 to collect the testes for the proposed evaluations.

The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

**2.2. Euthanasia of the rats and obtainment of testes and right epididymis**

Male rats on PND 53 and 110 were euthanized following narcosis by $CO_2$ asphyxiation. On PND 53, both testes were collected and the right one was used for intratesticular testosterone determination and sperm counts. Left testis was utilized for immunohistochemical purposes. During sexual maturity, on PND 110, testes were collected and the right organ was used for intratesticular testosterone determination and antioxidant analyses. On the other hand, left gonad was utilized for histological and immunohistochemical analyses. Right epididymis was collected from the animals and sperm was obtained from proximal cauda of the organ and were used for ultrastructural evaluation.

**2.3. Testicular hormonal concentrations**

Testicular fluid was obtained by centrifugation (10000 x g, 5 minutes, 4ºC) of the right testis (without tunica albuginea) in a refrigerated device and was frozen at – 80ºC until the moment of hormonal determination. Intratesticular testosterone concentrations were determined by double-antibody radioimmunoassay and were measured using specific kits provided by MP Biomedicals (Orangeburgh, NY, USA). The lower limit of detection was 0.07 pg/mL and the intra-assay coefficient of variation was 4.0%.

**2.4. Sperm counts in the pubescent testis**

The weight of testicular parenchyma was obtained after obtaining testicular fluid discounting the weight of tube immediately after centrifugation. Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as described previously (Robb, Amann, & Killian, 1978), with the following adaptations: right testicular parenchyma from each pubescent animal was homogenized in 5 mL of NaCl 0.9% containing Triton X 100

0.5%, followed by sonication for 30 seconds. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), proceeding a quantification of mature spermatids. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. To obtain the number of mature spermatids per gram of testis and the relative DSP, the number of mature spermatids and the DSP were divided by the weight of the testicular parenchyma.

**2.5. Antioxidant activity**

**2.5.1. Homogenate protein determination**

Right testis from each sexually mature rat was homogenized separately in 50 mM Tris-HCl buffer solution (pH-7.4). The protein content in testis homogenate was determined by Bradford assay using bovine serum albumin as a standard (Bradford, 1976).

**2.5.2. Thiobarbituric acid reactive substances (TBARS) assay**

The level of lipid peroxidation was assessed using the TBARS assay (Draper & Hadley, 1990). Briefly, 200 µL of testis homogenate was mixed with 500 µL thiobarbituric acid 0.8%, 500 µL acetic acid buffer, 200 µL of SDS 8.1% and 100 µL of water. These samples were then incubated for 2 hours at 95°C and allowed to cool to room temperature. The absorbance was measured at 532nm. The amount of lipid peroxidation was expressed as µM TBARS/mg protein. All of the assays were performed in duplicate.

**2.5.3. Reduced glutathione (GSH) levels**

Reduced glutathione levels were determined following the procedures reported previously (Ellman, 1959). Briefly, the homogenates were precipitated by adding 100 µl of 30% trichloroacetic acid (TCA) to 500 µl of the homogenate and then centrifuged at 4000×g for 10 min. The reaction mixture contains 50 µl of the supernatant, 50 µl of 10 mM DTNB and 900 µl of 1 M potassium phosphate buffer, pH 7.4. Absorbance was read at 412 nm and GSH expressed as nmol GSH/mg protein using GSH as standard or a molar extinction coefficient of 13,100 $M^{-1}/cm^{-1}$. The assays were performed in duplicate.

### 2.5.4. Catalase activity assay

Catalase activity was determined spectrophotometrically based on the rate of decomposition of hydrogen peroxide by catalase (Aebi, 1984). One unit of the enzyme is defined as the amount of enzyme that decomposes 1 mmol hydrogen peroxide in 2 min at 25 °C. Briefly, 900 µl of 50 mM phosphate buffer was transferred into a cuvette, and then 35 µl of 0.3 M hydrogen peroxide solution was added. Subsequently, 15 µl of the testicular homogenate was added, and the change in absorbance after 2 min was recorded. Absorbance was read at 240 nm using an ultraviolet/ visible (UV/Vis) spectrophotometer. Catalase activity was expressed as units/milligram protein using a molar extinction coefficient of 43.6 $M^{-1}/cm^{-1}$. All of the assays were performed in duplicate.

### 2.5.5. Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was determined spectrophotometrically following the method previously described with minor modifications (Paglia & Valentine, 1967). The reaction mixture contains 880 µl of the system solution (0.15 mM NADPH, 1 mM GSH, 0.1 U/ml GR, 100 mM azide, in potassium phosphate buffer, pH 7.0), 20 µl of water, 150 µl of the sample and 100 µl of 0.4 mM hydrogen peroxide. Absorbance change per minute was read at 340 nm and GPx activity was expressed as µ mol NADPH/min/mg protein. The assays were performed in duplicate.

## 2.6. Histological procedures

Left testis from pubescent and sexually mature animals were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 µm (transversal sections). Sections obtained in silanized slides were used for immunohistochemical evaluations and TUNEL assay. Other three non-serial testicular sections were obtained with a distance of 50 µm among them and were stained with Periodic Acid – Schiff (PAS) and counterstained with hematoxylin.

To assess spermatogenesis dynamics, one hundred random tubular sections per animal (n = 7 animals⁄ group) in three non-consecutive testis cross-sections were classified into fourteen stages (from stage I to stage XIV), accordingly to the degree of development of acrosome and the association of different germ cell layers (Leblond & Clermont, 1952), under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at x200 magnification. The evaluation was performed in a blind assay and the figures were obtained using a light microscope Leica, coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows.

**2.7. Immunohistochemistry for Androgen Receptor (AR) on testis**

Testicular tissues from pubescent and adult animals were sectioned in 4 μm, allocated in silanized slides, dewaxed using xylene (Sigma-Aldrich®, USA), hydrated using decreasing concentrations of ethanol and were washed in phosphate buffer (PBS), followed by antigen recovery with citrate buffer (0.01M, pH 6.0) in a pressure cooker submitted to high temperatures for 45 minutes. Moreover, endogenous peroxidase was quenched in 3% of hydrogen peroxide in methanol for 15 minutes. Thus, the sections were incubated in 3% BSA for 1 hour to block unspecific bonds, washed with PBS buffer and then, were incubated overnight with the primary antibody anti-androgen receptor (AR N-20: sc-816, 1:100, from Santa Cruz Biotechnology, CA, USA). After incubating, the slides were washed with PBS buffer and during 1 hour, at room temperature, the sections were incubated with secondary antibody (Biotinylated Goat Anti-Rabbit Immunoglobulins, 1:100 – Vector Laboratories®, CA, USA). After this step, the slides were washed with PBS buffer and submitted to the avidin-biotin-peroxidase solution (Vectastain Standard ABC Kit – Vector Laboratories®, CA, USA) for 45 minutes. The slides were washed with PBS buffer and submitted to diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich®, MO, USA) for 2 minutes, then, the slides were washed in tap water and counterstained with Harris hematoxylin. Thereafter, the slides were dehydrated in 70%, 90% and 100% ethanol and immersed in xylene. Coverslips were mounted on the slides with Entellan®. Negative controls were performed without primary antibody and minimal background was found (data not shown). All analyses were carried out using a light microscope Leica, coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows. The intensity of immunostaining in the cells was classified as absent, weak, moderate or strong and was always compared to the controls.

**2.8. Immunohistochemistry for Estrogen Receptor Alpha (ESR1) on testis**

Testicular sections from male rats on PND 53 and 110 were sectioned in 4 μm, allocated in silanized slides, dewaxed using xylene (Sigma-Aldrich®, USA), hydrated using decreasing concentrations of ethanol and were washed in phosphate buffer (PBS), followed by antigen recovery with citrate buffer (0.01M, pH 6.0) under controlled temperature (37°C) in an incubator for 30 minutes. Furthermore, endogenous peroxidase was quenched in 3% of hydrogen peroxide in methanol for 15 minutes. Thus, the sections were incubated in 3% BSA for 1 hour to block unspecific bonds, washed with PBS buffer and then, were incubated overnight with primary antibody anti-estrogen receptor alpha (ERα HC-20: sc-543, 1:100, from Santa Cruz Biotechnology, CA, USA). After incubating, the slides were washed with PBS buffer and during 2 hours, at room temperature, the sections were incubated with secondary antibody (Biotinylated Horse Anti-Mouse Immunoglobulins, 1:100 – Vector Laboratories®, CA, USA). After this step, the slides were washed with PBS buffer and submitted to the avidin-biotin-peroxidase solution (Vectastain Standard ABC Kits – Vector Laboratories®, CA, USA) for 2 hours. The slides were washed with PBS buffer and submitted to diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich®, MO, USA) for 5 minutes, then, the slides were washed in tap water and counterstained with Harris hematoxylin. Thereafter, the slides were dehydrated in 70%, 90% and 100% ethanol and immersed in xylene. Coverslips were mounted on the slides with Entellan®. Negative controls were performed without primary antibody and minimal background was found (data not shown). All analyses were carried out using a light microscope Leica, coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows. The intensity of immunostaining in the cells was classified as absent, weak, moderate or strong and was always compared to the controls.

**2.9. TUNEL assay**

For evaluation of *in situ* DNA fragmentation, TUNEL assay was performed using a commercial kit (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit S7100, Chemicon, Temecula, CA). The manufacturer's instructions were properly followed. Testicular sections from pubescent and adult male rats were sectioned in 4 μm were allocated in silanized slides, dewaxed using xylene (Sigma-Aldrich®, USA), hydrated using decreasing concentrations of ethanol and were washed in PBS buffer. Sections were incubated with proteinase K (20 μg/mL) for 15 minutes, washed with PBS buffer and then, endogenous peroxidase was quenched with 3% of hydrogen peroxide in PBS buffer for 5 minutes. After washing, the sections were exposed to equilibration buffer followed by incubation with Terminal deoxynucleotidyl transferase (TdT) enzyme for 1 hour in an incubator at 37°C. The reaction was blocked with stop buffer, followed by washing of the sections with PBS buffer and incubation with anti-digoxigenin-peroxidase for 30 minutes at room temperature. The slides were washed with PBS buffer, and then, submitted to diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich®, MO, USA) for 3 minutes. After this step, testicular sections were counterstained with Harris hematoxylin, dehydrated in crescent concentrations of ethanol and immersed in xylene. Coverslips were mounted on the slides with Entellan®. Negative controls were performed without TdT enzyme and minimal background was found (data not shown). Testicular sections were evaluated in the relation to presence of marked cells in different categories of stages of seminiferous epithelium (stages I-VI, VII-VIII, IX-XIII and XIV) and the results were expressed as the percentage of seminiferous tubules with marked germ cells in each category of stages. All analyses were carried out using a light microscope Leica, coupled to a digital camera and a personal computer with the software Leica Qwin version 3 for Windows.

**2.10. Conventional transmission electron microscope**

Sperm collected from right epididymis (n=4/group) was diluted in 2 mL of human tubal fluid (HTF) modified medium (Spectrun 90126) and soon after, the sperm was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 24 hours at room temperature, followed by post-fixation in 1% osmium tetroxide in the same buffer for 2 hours, after obtaining a pellet by centrifugation. After washing in distilled water, the samples were contrasted with an aqueous solution of 0.5% uranyl acetate for 2 hours at room temperature, dehydrated in graded acetone series (50%, 70%, 90% and 100%) and embedded in Araldite resin. Ultra-thin sections were contrasted with uranyl acetate and lead citrate, and posteriorly all samples were analyzed in a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscope Center of Biosciences Institute of Botucatu (UNESP).

**2.11. Statistical analysis**

For comparison of the results among the experimental groups, two-way ANOVA was performed, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed on GraphPad Prism (version 5.00).

## 3. Results

### 3.1. Hormonal measurements at puberty and adulthood and sperm counts on pubertal testis

There was a diminution on intratesticular testosterone concentrations on rosuvastatin-exposed groups, at the both doses, during puberty and at adulthood when compared to the control group ($p < 0.05$) (Figure 1). On the other hand, ascorbic-acid-supplemented groups showed intratesticular testosterone concentrations similar to the controls (Figure 1). Rosuvastatin-treated groups exhibited a decreased mature spermatid number and reduced daily sperm production in a dose-dependent manner ($p < 0.05$) (Table 1). Ascorbic-acid-treated groups showed daily sperm production and mature spermatid number similar to the control group (Table 1).

### 3.2. Antioxidant status at adulthood

The group exposed to the dose of 10 mg of rosuvastatin exhibited lower glutathione peroxidase activity and increased levels of GSH, in comparison with control group. Statin-exposed groups, at the both doses, showed increased levels of testicular catalase activity and lipid peroxidation on sexual maturity ($p < 0.05$) (Figure 2). However, the groups treated with ascorbic acid did not show significant differences for glutathione peroxidase and catalase activity, as well as, GSH and lipid peroxidation levels when compared to controls (Figure 2).

### 3.3. Immunohistochemistry for androgen and estrogen-alpha receptors on testis

Testicular tissue showed Sertoli cells with strong or moderate nuclear staining for androgen receptors at stages I-VI, VII-VIII and XIV among the groups during puberty and adulthood (Figures 3 and 4). There was a diminished immunostaining in the nuclei of Sertoli cells (weak staining) at stages IX-XIII in the rosuvastatin-exposed groups in both periods, in comparison with controls, which exhibited moderate staining (Figures 3 and 4). Leydig cells showed similar nuclear staining among the groups (Figures 3 and 4). Ascorbic-acid-supplemented groups showed immunostaining similar to the control group (Figures 3 and 4).

On puberty, there was similar strong cytoplasmic immunostaining for estrogen receptor-alpha in round and elongated spermatids among the groups (Figures 5 and 6). Furthermore, Leydig cells showed strong staining in their nuclei among the groups (Figures 5 and 6). On PND 110, it was shown a weaker cytoplasmic immunostaining on round and elongated spermatids in the group exposed to the higher dose of statin when compared to controls, which exhibited a moderate staining (Figures 5 and 6). Additionally, the group exposed to the dose of 10 mg of statin also showed weaker cytoplasmic and nuclear staining in Leydig cells (Figures 5 and 6). Ascorbic-acid-treated groups presented a strong immunostaining for estrogen receptor-alpha like controls (Figures 5 and 6).

**3.4. Testicular cell death and staging the cycle of seminiferous epithelium**

Pubertal testis exhibited an increased frequency of germ cell death (spermatogonias and spermatocytes) at stages I-VI, in both statin doses, in comparison with control group ($p < 0.05$) (Figure 7 and Table 2). Moreover, there was also augmented frequency of germ cell death at stages IX-XIII in the higher dose of rosuvastatin when compared to controls ($p < 0.05$) (Figure 7 and Table 2). The ascorbic-acid-supplemented groups exhibited a frequency of germ cell death like controls (Figure 7 and Table 2). Leydig cells did not show a TUNEL-positive staining (Figure 7).

During sexual maturity, it was shown an augmented rate of germ cell death (spermatogonias and spermatocytes) at stages I-VI in the groups exposed to 3 or 10 mg of statin ($p < 0.05$) (Figure 8 and Table 3). Additionally, the frequency of germ cell death was higher at stages IX-XIII and XIV in the group treated with the higher dose of rosuvastatin ($p < 0.05$) (Figure 8 and Table 3). Ascorbic-acid-supplemented groups presented frequency of germ cell death like control group (Figure 8 and Table 3). Leydig cells did not exhibit a TUNEL-positive staining (Figure 8).

On Figure 9, it is shown each stage of a control rat seminiferous epithelium and morphological appearance of interstitial tissue in PAS-stained sections used as the basis for staging and identification of histopathological lesions on the interstitial tissue. Staging the cycle of seminiferous epithelium showed no differences among the groups for each one of the fourteen stages of rat seminiferous epithelium ($p > 0.05$) (Table 4). Interstitial tissue morphology was similar among the groups (data not shown).

**3.5. Ultrastructural morphology of rat sperm**

Control and ascorbic-acid-supplemented groups showed resembling sperm ultrastructure among them, therefore presenting a sperm head with a dense nucleus and an acrosome above it; mitochondria around of the outer dense fibers and axoneme in the midpiece; and presence of fibrous sheath, outer dense fibers and axoneme in the principal peace (Figure 10). On the other hand, the group exposed to the lower dose of statin exhibited less dense and disorganized outer dense fibers, in comparison with control animals (Figure 10). Furthermore, the higher dose of rosuvastatin showed an absence of outer dense fibers or less dense and disorganized outer dense fibers, and regions without mitochondria in sperm midpiece, in comparison with control group (Figure 10).

## 4. Discussion

This study is based on the fact that pediatric obesity is currently increasing (Jiménez & Ferre, 2011) and statins are recommended for lipid-lowering purposes and to prevent cardiovascular risk (Ludman, Venugopal, Yellon, & Hausenloy, 2009; Tandon, Bano, Khajuria, Parihar, & Gupta, 2005). Although we have used non-obese juvenile rats in this experimental protocol, the study simulates the human exposure to rosuvastatin during pre-puberty, considering that statins exhibit benefit effects, named pleiotropic effects, in individuals that use the medication (Park, 2013; Tandon et al., 2005).

Previous studies demonstrated that statin exposure reduce serum testosterone concentration (Hsieh & Huang, 2016; Leite, Figueiredo, Pacheco, et al., 2017; Leite, Figueiredo, Sanabria, et al., 2017), delay puberty onset, reduce sperm motility and increase sperm DNA damage (Leite et al., 2014; Leite, Figueiredo, Pacheco, et al., 2017; Leite, Figueiredo, Sanabria, et al., 2017). On the other hand, ascorbic acid seems to ameliorate reproductive damage in different studies due to its role in male reproduction, improving testosterone levels and antioxidant status, besides to diminish oxidative stress (Fernandes, Fernandez, et al., 2011; Fernandes, Gerardin, et al., 2011; Leite, Figueiredo, Sanabria, et al., 2017). Thus, this study aimed to assess the possible testicular damage promoted by pre-pubertal exposure to rosuvastatin and the role of ascorbic acid supplementation on the attempt to alleviate the possible testicular impairment.

Puberty is a critical period in which important morphological alterations are occurring in mammals, besides neuroendocrine interactions, such as the reactivation of hypothalamic-pituitary-gonadal (HPG) axis (Stoker et al., 2000; Zawatski & Lee, 2013). On pubertal period, spermatogenesis and steroidogenesis were not completely established, thus testicular tissue is susceptible to the exposure to endocrine disruptors, especially those that promote androgen depletion in this critical period (Blystone, Lambright, Furr, Wilson, & Gray, 2007; Favareto, Fernandez, da Silva, Anselmo-Franci, & Kempinas, 2011; Johnson, Welsh, & Wilker, 1997). Reproductive changes during the pubertal period may even affect reproduction during sexual maturity (Mantovani & Fucic, 2014; Juliana E. Perobelli et al., 2013). Indeed, in this study, it was observed a diminished intratesticular testosterone concentration in both periods, puberty and adulthood, in the groups exposed to rosuvastatin, probably due to the inhibition of HMG-CoA reductase, an important enzyme in the biosynthesis of steroid precursors, named cholesterol molecules. Ascorbic acid was capable to restore intratesticular testosterone concentrations in the co-exposed groups during puberty and sexual maturity.

Previous studies demonstrated that statins reduce androgen production by diminishing the expression of *CYP17A1* and decreasing isoprenylation in theca-interstitial cells culture (Ortega et al., 2012, 2014). Contrary to this, ascorbic acid is able to increase androgen production by augmenting LH receptors expression in Leydig cells and stimulating steroidogenic enzymes 3β-HSD, 17β-HSD and StAR protein in the testis (Harikrishnan et al., 2013; Radhakrishnakartha, Appu, & Indira, 2014).

Daily sperm production in rats is increasing since peri-puberty and only reaches highest sperm production at 75 days of age, approximately (Perobelli et al., 2012; Robb et al., 1978). Assessing the sperm production is a way to identify possible testicular toxic effects due to exposure to an endocrine disruptor (Perreault & Cancel, 2001). Statin-treated groups showed a decreased number of mature spermatids and diminished daily sperm production in a dose-dependent manner. On the other hand, ascorbic acid was able to ameliorate the daily sperm production, probably due to its ability on increasing testosterone levels. It is known that androgens are necessary for the maturation of germinal epithelium and the maintenance of spermatogenesis (Adamopoulos, Vassilopoulos, Kontogeorgos, & Kapolla, 1990). Additionally, previous study showed a decrease in the degree of maturation of seminiferous epithelium in statin-exposed rats, suggesting a delay of testicular maturation (Leite, Figueiredo, Sanabria, et al., 2017).

Androgens bind to their androgen receptors in the cytoplasm and then, these hormone-receptor complexes are translocated to the nucleus where they will regulate gene expression (Collins et al., 2003; Ungefroren, Ivell, & Ergun, 1997), especially those important for maturation of male reproductive system and fertility (Collins et al., 2003). Decreased androgen concentrations are related to lower nuclear staining for androgen receptors in Sertoli cells at stages IX-XIII (Leite et al., 2014). In this study, there was also a reduced nuclear immunostaining for androgen receptors in Sertoli cells at stages IX-XIII, at the both ages, in the groups exposed to rosuvastatin. These findings are associated with androgen depletion observed in statin-treated groups in both periods. Since ascorbic acid improves testosterone levels, we hypothesized that the expression of androgen receptors in Sertoli cells remains similar to the controls in consequence of a normal androgen supply by Leydig cells.

Sertoli cells need sufficient androgen stimulation to secrete proteins related to the maintenance and survival of germ cells (De Gendt et al., 2004; Stanton et al., 2012). When there is androgen depletion there are increased DNA damage and raised germ cell death in the testis due to the diminished secretion of survival proteins by Sertoli cells (Stanton et al., 2012). Moreover, the diminished levels of intermediate isoprenoids, mediated by inhibition of HMG-CoA reductase, are also related to cell death and diminished cell growth (Adam & Laufs, 2008; Ortega et al., 2012). Indeed, there was an augmentation in germ cell death in rosuvastatin-treated groups during puberty (stages I-VI in the lower dose of statin and stages I-VI and IX-XIII in the higher dose) and adulthood (stages I-VI in the dose of 3 mg and stages I-VI, IX-XIII and XIV in the dose of 10 mg). This increased cell death may justify the lower sperm production during puberty observed in this study, and the reduced sperm counts at adulthood in consequence of prepubertal exposure to the statin, as previously reported (Leite, Figueiredo, Pacheco, et al., 2017). Ascorbic acid, via improving testosterone levels and probably restoring Sertoli cells secreting function, was able to prevent the increased germ cell death in the co-exposed groups.

It is reported that estrogens play a crucial role in testicular tissue, acting in the germ cell maturation during spermatogenesis (D'Souza et al., 2005). Estrogens mainly play their role through binding to their cytoplasmic receptors (ERS1 or ERS2) forming hormones-receptors complexes that are translocated to the nucleus, where they modulate gene expression (Carreau et al., 2011). Previous studies have demonstrated that ERS1 knockout mice show infertility at adulthood (Shibayama et al., 2001) and fluid accumulation in the testis, an abnormal concentration of sperm and compromised sperm maturation in the caput of epididymis (Hess, 2014). Furthermore, a decreased ERS1 expression is also related to increased sperm abnormalities, decreased sperm motility (Couse & Korach, 1999; Guerra et al., 2016) and reduced sperm counts (Couse & Korach, 1999). In this study, the control group exhibited similar moderate cytoplasmic staining for ERS1 in the round and elongated spermatids and in Leydig cells, as previously described (Guerra et al., 2016). Moreover, the Leydig cells showed a strong nuclear staining for ERS1, which is in accordance with a previous study (Pelletier, Labrie, & Labrie, 2000). The experimental groups showed similar ERS1staining like controls during puberty, however, at adulthood, the group exposed to the higher dose of rosuvastatin exhibited weaker ERS1 cytoplasmic staining in Leydig cells and in the round and elongated spermatids, as well as, lower nuclear staining in Leydig cells, in comparison with the other groups. This may be related to a possible hormonal deregulation, especially an estradiol concentration imbalance, in this group.

In addition, staging the cycle of seminiferous epithelium is an important assessment that enables to identify the failure on acrosome development and the loss of entire layers of germ cells in histopathological studies, besides to allow the determination of specific steps of spermatid development (Meistrich & Hess, 2013). However, in this study, rosuvastatin exposure did not affect the spermatogenesis kinetics, which was assessed through staging germinal epithelium in testicular sections. All the groups showed similar frequencies of each stage of the seminiferous epithelium.

Leite et al. (2017) have shown decreased sperm motility in rosuvastatin exposed rats. Decreased sperm motility is often associated with ultrastructural abnormalities in the flagellum (Medina et al., 2017) or compromised sperm maturation during sperm transit time (Perreault & Cancel, 2001). Flagellum outer dense fibers, when in an organized arrangement, allow the elastic recoil of the axoneme and supplies elastic recoil force, thus preventing its rupture during the transit through viscoelastic media (Inaba, 2003). Moreover, the mitochondrial sheath in the midpiece is the source of energy for sperm motility and when there is a lower number of mitochondria, then, there is diminished energy production for the movement of the sperm flagellum (Medina et al., 2017). In this work, we observed that both rosuvastatin-exposed groups presented alterations in outer dense fibers (disorganized or less dense fibers in both groups, and absence in the higher dose); additionally the higher dose of statin also showed regions without mitochondria in the sperm midpiece. These results suggest that flagellum and sperm midpiece ultrastructural damages may be, at least, two of the reasons for reduced sperm motility in statin-exposed rats, as reported previously.

Moreover, studies have demonstrated that statins may interfere with mitochondrial function and diminish coenzyme Q10 levels, an important compound for mitochondrial bioenergetics (Apostolopoulou, Corsini, & Roden, 2015; Broniarek & Jarmuszkiewicz, 2016; Littarru & Tiano, 2007). In consequence of statin exposure, the main mitochondrial dysfunctions are related to the diminished protein isoprenylation and inhibition of respiratory chain complexes due to the role of coenzyme Q10 as an essential electron carrier (Broniarek & Jarmuszkiewicz, 2016).

In rodents, as well as in humans, the increase in the production of reactive oxygen and nitrogen species may cause modifications on DNA and proteins and lead to augmented levels of lipid peroxidation substances, overcoming the endogenous antioxidant status in the cells and tissues (Mouthuy et al., 2016). Enzymes such as catalase and glutathione peroxidase, form the cellular antioxidant system that acts decomposing hydrogen peroxide, thus preventing the excessive lipid peroxidation and avoiding cellular impairment (Aebi, 1984; Mouthuy et al., 2016).

Indeed, catalase and glutathione peroxidase are the main enzymes involved in detoxification of hydrogen peroxide and catalase is the first enzyme recruited when there are high concentrations of this highly reactive molecule (Baud, 2004). Previous study has reported increased catalase activity in pubertal testis from rats exposed to 3 mg of statin (Leite, Figueiredo, Sanabria, et al., 2017). In fact, in this study, catalase activity and lipid peroxidation was increased in both rosuvastatin-exposed groups during adulthood, suggesting increased levels of hydrogen peroxide and increased oxidative stress.

Endogenous GSH levels contribute to the maintenance of cell homeostasis by eliminating free radicals in consequence of physical or chemical injuries (Inal, Akgün, & Kahraman, 2003). In this work, there was an augmentation of GSH levels and a reduced activity of glutathione peroxidase in the group exposed to the higher dose of statin, indicating that the major source of detoxification, in this study, is mediated by increased catalase activity.

Ascorbic acid is one of the small molecules that contribute to the formation of the antioxidant system with the participation of other molecules, such as glutathione, tocopherol and tocotrienol (Mouthuy et al., 2016). Additionally, ascorbic acid is able to raise the antioxidant status and is responsible for up 65% of antioxidant activity on semen (Fernandes, Fernandez, et al., 2011; Leite, Figueiredo, Sanabria, et al., 2017; Makker, Agarwal, & Sharma, 2009). The results indicate that pre-pubertal supplementation with ascorbic acid is able to diminish oxidative stress and restore antioxidant status at adulthood, by acting on the maturation of antioxidant system during puberty and increasing antioxidant status during this period, as previously shown (Leite, Figueiredo, Sanabria, et al., 2017).

## 5. Conclusion

In summary, pre-pubertal exposure to rosuvastatin impaired steroid hormone receptors distribution in the testis, promoted androgen depletion and sperm ultrastructural changes, increased testicular oxidative stress and augmented germ cell death. Ascorbic acid, as an important antioxidant compound, was able to ameliorate the antioxidant status and reduce oxidative stress, by decreasing lipid peroxidation, besides to restore testicular structural damage and lower androgen concentrations promoted by statin exposure.

## 6. Conflicts of interest

The authors declare that there are no conflicts of interest.

## 7. Acknowledgments

The authors are grateful to São Paulo Research Foundation (FAPESP) (Grant #013/22495-1), National Council for Scientific and Technological Development (CNPq) (Grant 308842/2013-8) and Coordination for the Improvement of Higher Education Personnel (CAPES) for their financial support, to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for his excellent technical assistance and to Dr. Ruither de Oliveira Gomes Carolino of the Department of Morphology, Physiology and Basic Pathology of the School of Dentristy of Ribeirão Preto, USP, for the hormones measurements.

## 8. References

Adam, O., & Laufs, U. (2008). Antioxidative effects of statins. *Archives of Toxicology*. https://doi.org/10.1007/s00204-008-0344-4

Adamopoulos, D. A., Vassilopoulos, P., Kontogeorgos, L., & Kapolla, N. (1990). Germinal epithelium changes in sexually immature rabbits treated with intratesticular testosterone implants. *Andrologia*, *22*, 557–565.

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, *105*(1947), 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Agarwal, A., Prabhakaran, S. A., & Said, T. M. (2005). Prevention of Oxidative Stress Injury to Sperm. *Journal of Andrology*, *26*(6), 654–660. https://doi.org/10.2164/jandrol.05016

Apostolopoulou, M., Corsini, A., & Roden, M. (2015). The role of mitochondria in statin-induced myopathy. *European Journal of Clinical Investigation*, *45*(7), 745–754. https://doi.org/10.1111/eci.12461

Baud, O. (2004). Glutathione peroxidase-catalase cooperativity Is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *The Journal of Neuroscience*, *24*(7), 1531–1540. https://doi.org/10.1523/JNEUROSCI.3989-03.2004

Blystone, C. R., Lambright, C. S., Furr, J., Wilson, V. S., & Gray, L. E. (2007). Iprodione delays male rat pubertal development, reduces serum testosterone levels, and decreases ex vivo testicular testosterone production. *Toxicology Letters*, *174*(1–3), 74–81. https://doi.org/10.1016/j.toxlet.2007.08.010

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Broniarek, I., & Jarmuszkiewicz, W. (2016). Statins and mitochondria. *Postępy Biochemii*, *62*(2), 77–84.

Carreau, S., Bois, C., Zanatta, L., Silva, F. R. M. B., Bouraima-Lelong, H., & Delalande, C. (2011). Estrogen signaling in testicular cells. *Life Sciences*, *89*, 584–587. https://doi.org/10.1016/j.lfs.2011.06.004

Collins, L. L., Lee, H. J., Chen, Y. T., Chang, M., Hsu, H. Y., Yeh, S., & Chang, C. (2003). The androgen receptor in spermatogenesis. *Cytogenetic and Genome Research*, *103*(3–4), 299–301. https://doi.org/10.1159/000076816

Cook, S., & Kavey, R. E. W. (2011). Dyslipidemia and pediatric obesity. *Pediatric Clinics of North America*, *58*(6), 1363–1373. https://doi.org/10.1016/j.pcl.2011.09.003

Couse, J. F., & Korach, K. S. (1999). Estrogen receptor null mice: What we learned and where will they lead us? *Endocrine Reviews*, *20*(3), 358–417. https://doi.org/10.1210/edrv.20.3.0370

D'Souza, R., Gill-Sharma, M. K., Pathak, S., Kedia, N., Kumar, R., & Balasinor, N. (2005). Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. *Molecular and Cellular Endocrinology*, *241*(1–2), 41–48. https://doi.org/10.1016/j.mce.2005.04.011

De Gendt, K., Swinnen, J. V, Saunders, P. T. K., Schoonjans, L., Dewerchin, M., Devos, A., … Verhoeven, G. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(5), 1327–1332. https://doi.org/10.1073/pnas.0308114100

Draper, H. H., & Hadley, M. (1990). Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology*, *186*, 421–431. https://doi.org/10.1016/0076-6879(90)86135-I

Ellman, G. L. (1959). Tissue Sulfhydryl Groups. *Archives of Biochemistry and Biophysics*, *82*, 70–77. https://doi.org/https://doi.org/10.1016/0003-9861(59)90090-6

Eskenazi, B., Kidd, S. A., Marks, A. R., Sloter, E., Block, G., & Wyrobek, A. J. (2005). Antioxidant intake is associated with semen quality in healthy men. *Human Reproduction*, *20*(4), 1006–1012. https://doi.org/10.1093/humrep/deh725

Favareto, A. P. A., Fernandez, C. D. B., da Silva, D. A. F., Anselmo-Franci, J. A., & Kempinas, W. D. G. (2011). Persistent Impairment of Testicular Histology and Sperm Motility in Adult Rats Treated with Cisplatin at Peri-Puberty. *Basic and Clinical Pharmacology and Toxicology*, *109*(2), 85–96. https://doi.org/10.1111/j.1742-7843.2011.00688.x

Fernandes, G. S. A., Fernandez, C. D. B., Campos, K. E., Damasceno, D. C., Anselmo-Franci, J. A., & Kempinas, W. D. G. (2011). Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. *Reproductive Biology and Endocrinology : RB&E*, *100*, 1–9. https://doi.org/10.1186/1477-7827-9-100

Fernandes, G. S. A., Gerardin, D. C. C., Assumpção, T. A., Campos, K. E., Damasceno, D. C., Pereira, O. C. M., & Kempinas, W. D. G. (2011). Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats? *Pharmacological Reports*, *63*(4), 983–991.

Guerra, M. T., Sanabria, M., Leite, G. A. A., Borges, C. S., Cucielo, M. S., Anselmo-franci, J. A., … Kempinas, W. G. (2016). Maternal Exposure to Butyl Paraben Impairs Testicular Structure and Sperm Quality on Male Rats. *Environmental Toxicology*, *32*(4), 1273–1289. https://doi.org/10.1002/tox

Harikrishnan, R., Abhilash, P. A., Syam Das, S., Prathibha, P., Rejitha, S., John, F., … Indira, M. (2013). Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male guinea pigs. *The British Journal of Nutrition*, *110*(4), 689–98. https://doi.org/10.1017/S0007114512005739

Hess, R. A. (2014). Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segment of the epididymis. *Spermatogenesis*, *4*(2), 1–15. https://doi.org/10.4161/21565562.2014.979103

Holdgate, G. A., Ward, W. H. J., & McTaggart, F. (2003). Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. *Biochemical Society Transactions*, *31*(3), 528–531. https://doi.org/10.1042/BST0310528

Hsieh, C. J., & Huang, B. (2016). Rosuvastatin decreases testosterone levels but not sexual function in men with type 2 diabetes. *Diabetes Research and Clinical Practice*, *120*(10), 81–88. https://doi.org/10.1016/j.diabres.2016.07.026

Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. *Zoological Science*, *20*, 1043–1056. https://doi.org/https://doi.org/10.2108/zsj.20.1043

Inal, M. E., Akgün, A., & Kahraman, A. (2003). The effects of exogenous glutathione on reduced glutathione level, glutathione peroxidase and glutathione reductase activities of rats with different ages and gender after whole-body Γ-irradiation. *Journal of the American Aging Association*, *26*(3–4), 55–58. https://doi.org/10.1007/s11357-003-0005-8

Istvan, E. S., & Deisenhofer, J. (2001). Structural mechanism for statin inhibition of HMG-CoA reductase. *Science*, *292*(5519), 1160–1164. https://doi.org/10.1126/science.1059344

Izar, M. C. D. O., Fonseca, M. I. H., & Fonseca, F. A. H. (2011). Dyslipidemias. *Revista Brasileira de Medicina*, *68*(12), 59–73.

Jiménez, E. G., & Ferre, J. A. (2011). Estatinas : Características y Efectos sobre el Control Lipídico en el Niño y Adolescente Obeso. *Revista Clínica de Medicina de Familia*, *4*(1), 69–75.

Johnson, L., Welsh, T. H. J., & Wilker, C. E. (1997). Anatomy and physiology of the male reproductive system and potential targets of toxicants. In K. Boekleheide, R. E. Chapin, P. B. Hoyer, & C. Harris (Eds.), *Comprehensive Toxicology* (pp. 5–61). New York: Pergamon.

Kwiterovich, P. O. (2008a). Clinical and laboratory assessment of cardiovascular risk in children: Guidelines for screening, evaluation, and treatment. *Journal of Clinical Lipidology*, *2*(4), 248–266. https://doi.org/10.1016/j.jacl.2008.06.003

Kwiterovich, P. O. (2008b). Recognition and management of dyslipidemia in children and adolescents. *Journal of Clinical Endocrinology and Metabolism*, *93*(11), 4200–4209. https://doi.org/10.1210/jc.2008-1270

Leblond, C. P., & Clermont, Y. (1952). Spermiogenesis of rat, mouse,hamster and guinea pig as revealed by the “periodic acid-fuchsin sulfurous acid” technique. *American Journal of Anatomy*, *90*(2), 167–215. https://doi.org/10.1002/aja.1000900202

Leite, G. A. A., Figueiredo, T. M., Pacheco, T. L., Sanabria, M., Silva, P. V., Fernandes, F. H., & Kempinas, W. D. G. (2017). Vitamin C partially prevents reproductive damage in adult male rats exposed to rosuvastatin during prepuberty. *Food and Chemical Toxicology*, *109*(1), 272–283. https://doi.org/https://doi.org/10.1016/j.fct.2017.09.003

Leite, G. A. A., Figueiredo, T. M., Sanabria, M., Dias, A. F. M. G., Silva, P. V., Martins Junior, A. da C., … Kempinas, W. D. G. (2017). Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty. *Reproductive Toxicology*, *73*, 328–338. https://doi.org/10.1016/j.reprotox.2017.07.006

Leite, G. A. A., Rosa, J. de L., Sanabria, M., Cavariani, M. M., Franci, J. A. A., Pinheiro, P. F. F., & Kempinas, W. D. G. (2014). Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty. *Reproductive Toxicology*, *44*(4), 93–103. https://doi.org/10.1016/j.reprotox.2014.01.004

Littarru, G. P., & Tiano, L. (2007). Bioenergetic and antioxidant properties of coenzyme Q10: Recent developments. *Molecular Biotechnology*, *37*(1), 31–37. https://doi.org/10.1007/s12033-007-0052-y

Ludman, A., Venugopal, V., Yellon, D. M., & Hausenloy, D. J. (2009). Statins and cardioprotection - More than just lipid lowering? *Pharmacology and Therapeutics*, *122*(1), 30–43. https://doi.org/10.1016/j.pharmthera.2009.01.002

Makker, K., Agarwal, A., & Sharma, R. (2009). Oxidative stress & male infertility. *The Indian Journal of Medical Research*, *129*(4), 357–367.

Mantovani, A., & Fucic, A. (2014). Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. *Reproductive Toxicology*, *44*(4), 15–22. https://doi.org/10.1016/j.reprotox.2013.06.002

McTaggart, F. (2003). Comparative pharmacology of rosuvastatin. *Atherosclerosis Supplements*, *4*(1), 9–14. https://doi.org/10.1016/S1567-5688(03)00004-7

Medina, F. M., Arrieta, M. C., Villafañe, M. N., Klyver, S. M. R., Odstrcil, I. M. A. O., & Gonzaléz, M. E. (2017). Early signs of toxicity in testes and sperm of rats exposed to low cadmium doses. *Toxicology and Industrial Health*, *33*(7), 1–12. https://doi.org/10.1177/0748233716689524

Meistrich, M. L., & Hess, R. A. (2013). Assessment of spermatogenesis through staging of seminiferous tubules. *Methods in Molecular Biology*, *927*, 299–307. https://doi.org/10.1007/978-1-62703-038-0

Mouthuy, P.-A., Snelling, S. J. B., Dakin, S. G., Milković, L., Gašparović, A. C., Carr, A. J., & Žarković, N. (2016). Biocompatibility of implantable materials: An oxidative stress viewpoint. *Biomaterials*, *109*, 55–68. https://doi.org/10.1016/j.biomaterials.2016.09.010.

Mukhopadhyay, P. K., Dey, A., Mukherjee, S., & Pradhan, N. K. (2013). The effect of coadministration of alpha-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. *Journal of Basic and Clinical Physiology and Pharmacology*, *24*(4), 245–253. https://doi.org/10.1515/jbcpp-2012-0039

Olsson, A. G., McTaggart, F., & Raza, A. (2002). Rosuvastatin: a highly effective new HMG-CoA reductase inhibitor. *Cardiovascular Drug Reviews*, *20*(4), 303–328. https://doi.org/10.1111/j.1527-3466.2002.tb00099.x

Ortega, I., Cress, A. B., Wong, D. H., Villanueva, J. a, Sokalska, A., Moeller, B. C., … Duleba, A. J. (2012). Simvastatin reduces steroidogenesis by inhibiting Cyp17a1 gene expression in rat ovarian theca-interstitial cells. *Biology of Reproduction*, *86*(1), 1–9. https://doi.org/10.1095/biolreprod.111.094714

Ortega, I., Villanueva, J. A., Wong, D. H., Cress, A. B., Sokalska, A., Stanley, S. D., & Duleba, A. J. (2014). Resveratrol potentiates effects of simvastatin on inhibition of rat ovarian theca-interstitial cells steroidogenesis. *Journal of Ovarian Research*, *21*(7), 1–8.

https://doi.org/10.1186/1757-2215-7-21

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Park, A. (2013). Statins have few side effects, but should more people be taking them? *Time*. Retrieved from http://healthland.time.com/2013/07/10/statins-have-few-side-effects-but-should-more-people-be-taking-them/

Pelletier, G., Labrie, C., & Labrie, F. (2000). Localization of estrogen receptor alpha, estrogen receptor beta and androgen receptors in the rat reproductive organ. *Journal of Endocrinology*, *165*, 359–370.

Perobelli, J. E., Alves, T. R., de Toledo, F. C., Fernandez, C. D. B., Anselmo-Franci, J. A., Klinefelter, G. R., & Kempinas, W. D. G. (2012). Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. *Reproductive Toxicology*, *33*(3), 308–315. https://doi.org/10.1016/j.reprotox.2011.12.011

Perobelli, J. E., Patrão, M. T. C. C., Fernandez, C. D. B., Sanabria, M., Klinefelter, G. R., Avellar, M. C. W., & Kempinas, W. D. G. (2013). Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. *Reproductive Toxicology*, *38*(7), 65–71. https://doi.org/10.1016/j.reprotox.2013.03.004

Perreault, S. D., & Cancel, A. M. (2001). Significance of incorporating measures of sperm production and function into rat toxicology studies. *Reproduction*, *121*(2), 207–216. https://doi.org/10.1530/reprod/121.2.207

Pons-Rejraji, H., Brugnon, F., Sion, B., Maqdasy, S., Gouby, G., Pereira, B., … Tauveron, I. (2014). Evaluation of atorvastatin efficacy and toxicity on spermatozoa, accessory glands and gonadal hormones of healthy men: a pilot prospective clinical trial. *Reproductive Biology and Endocrinology : RB&E*, *12*(1), 65. https://doi.org/10.1186/1477-7827-12-65

Radhakrishnakartha, H., Appu, A. P., & Indira, M. (2014). Ascorbic acid supplementation enhances recovery from ethanol induced inhibition of Leydig cell steroidogenesis than abstention in male guinea pigs. *European Journal of Pharmacology*, *723*(1), 73–79. https://doi.org/10.1016/j.ejphar.2013.12.010

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *The FASEB Journal : Official Publication of the Federation of American*

*Societies for Experimental Biology*, *22*(3), 659–661. https://doi.org/10.1096/fj.07-9574LSF

Robb, G. W., Amann, R. P., & Killian, G. J. (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *Journal of Reproduction and Fertility*, *54*(1), 103–107. https://doi.org/10.1530/jrf.0.0540103

Ross, J. L. (2016). Statins in the Management of Pediatric Dyslipidemia. *Journal of Pediatric Nursing*, *31*(6), 723–735. https://doi.org/10.1016/j.pedn.2016.07.004

Shibayama, T., Fukata, H., Sakurai, K., Adachi, T., Komiyama, M., Iguchi, T., & Mori, C. (2001). Neonatal exposure to genistein reduces expression of estrogen receptor alpha and androgen receptor in testes of adult mice. *Endocrine Journal*, *48*(6), 655–663. https://doi.org/https://doi.org/10.1507/endocrj.48.655

Shrilatha, B., & Muralidhara. (2007). Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. *Reproductive Toxicology*, *23*(4), 578–587. https://doi.org/10.1016/j.reprotox.2007.02.001

Sönmez, M., Turk, G., & Yuce, A. (2005). The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology*, *63*, 2063–2072. https://doi.org/10.1016/j.theriogenology.2004.10.003

Stanton, P. G., Sluka, P., Foo, C. F. H., Stephens, A. N., Smith, A. I., Mclachlan, R. I., & Donnell, L. O. (2012). Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation: Impact on meiotic cells. *PLoS One*, *7*(7), 1–19. https://doi.org/10.1371/journal.pone.0041718

Stoker, T. E., Laws, S. C., Guidici, D. L., & Cooper, R. L. (2000). The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicological Sciences*, *58*(1), 50–59. https://doi.org/10.1093/toxsci/58.1.50

Tandon, V., Bano, G., Khajuria, V., Parihar, A., & Gupta, S. (2005). Pleiotropic effects of statins. *Indian Journal of Pharmacology*, *37*(2), 77–85. https://doi.org/10.5772/23502

Ungefroren, H., Ivell, R., & Ergun, S. (1997). Region-specific expression of the androgen receptor in the human epididymis. *Molecular Human Reproduction*, *3*(11), 933–940.

Vaughan, C. J., & Gotto Jr, A. M. (2004). Update on Statins: 2003. *Circulation*, *110*(7), 886–892. https://doi.org/10.1161/01.CIR.0000139312.10076.BA

Zawatski, W., & Lee, M. M. (2013). Male pubertal development: Are endocrine-disrupting compounds shifting the norms? *Journal of Endocrinology*, *218*(2), 1–12. https://doi.org/10.1530/JOE-12-0449

**Figure Captions**

**Figure 1.** Intratesticular testosterone concentrations (ng/ml) in male rats from the different experimental groups at 53 and 110 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate significant differences among the groups.

**Figure 2.** Lipid peroxidation, GSH levels, catalase and glutathione peroxidase activity in the testis from the different experimental groups on post-natal day (PND) 110. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate significant differences among the groups.

**Figure 3.** Immunostaining of AR in pubertal rat testis from the different experimental groups ($n$ = 5/group). Note the diminished nuclear staining in the Sertoli cells at stages IX-XIII (arrows). Scale bar = 50µm.

**Figure 4.** Immunostaining of AR in adult rat testis from the different experimental groups ($n$ = 5/group). Note the diminished nuclear staining in the Sertoli cells at stages IX-XIII (arrows). Scale bar = 50µm.

**Figure 5.** Immunostaining of ERS1 in pubertal rat testis from the different experimental groups ($n$ = 5/group). Scale bar = 50µm.

**Figure 6.** Immunostaining of ERS1 in adult rat testis from the different experimental groups ($n$ = 5/group). Note the diminished cytoplasmic staining in Leydig cells, round, and elongated spermatids at all stages (arrows). Observe the reduced nuclear staining in Leydig cells. Scale bar = 50µm.

**Figure 7.** Photomicrography of TUNEL staining in testicular sections from the different experimental groups on PND 53. Observe the presence of TUNEL-marked germ cells (arrows) in the seminiferous epithelium of rosuvastatin-exposed groups. Scale bar = 50µm.

**Figure 8.** Photomicrography of TUNEL staining in testicular sections from the different experimental groups on PND 110. Observe the presence of TUNEL-marked germ cells (arrows) in the seminiferous epithelium of rosuvastatin-exposed groups. Scale bar = 50µm.

**Figure 9.** Photomicrography of stages of the cycle of seminiferous epithelium used as the base for staging and interstitial tissue from an adult control rat stained with Periodic Acid-Schiff (PAS) and Hematoxylin. Scale bar = 50µm.

**Figure 10.** Transmission electron microscope micrographs of rat sperm from different groups. (A) Longitudinal section of sperm head of a control rat; (B) Longitudinal section of sperm head and midpiece of a control rat; (C) Transversal section of sperm midpiece of a control rat; (D) Transversal section of sperm principal piece of a control rat; (E-G) Transversal section of sperm midpiece of a rat from the group AA, 3mg+AA and 10mg+AA, respectively; (H-I) Transversal section of sperm midpiece of a rat from the group 3mg and 10mg showing disorganized or less dense ODF (asterisks); (J) Transversal section of sperm midpiece of a rat from the group 10mg showing absence of one ODF (arrowhead); (K-L) Transversal and longitudinal section of sperm midpiece of a rat from the group 10mg showing absence of a mitochondrion (arrows), respectively. nu: nucleus; ac: acrosome; pm: plasma membrane, mi: mitochondria; ax: axoneme; ODF: outer dense fibers; FS = fibrous sheath. Scale bar = 1µm, 500nm and 200nm.

**Table 1.** Sperm counts in the testis from the different experimental groups on post-natal day (PND) 53.

| | Experimental Groups (n = 10) | | | | | |
|---|---|---|---|---|---|---|
| **Sperm counts in the testis** | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Mature spermatid number ($10^6$/testis) | $79.06 \pm 4.24^{a}$ | $69.55 \pm 4.47^{ab}$ | $65.16 \pm 4.11^{b}$ | $75.12 \pm 5.12^{a}$ | $69.18 \pm 4.10^{ab}$ | $73.02 \pm 3.49^{ab}$ |
| Mature spermatid number ($10^6$/g testis) | $78.53 \pm 1.77^{a}$ | $71.37 \pm 4.19^{ab}$ | $59.05 \pm 3.53^{b}$ | $69.25 \pm 2.96^{ab}$ | $71.73 \pm 3.28^{ab}$ | $76.16 \pm 3.01^{a}$ |
| Daily sperm production ($10^6$/testis/day) | $12.96 \pm 0.69^{a}$ | $11.40 \pm 0.73^{ab}$ | $10.68 \pm 0.35^{b}$ | $12.31 \pm 0.84^{a}$ | $11.34 \pm 0.67^{ab}$ | $11.97 \pm 0.57^{ab}$ |
| Relative sperm production ($10^6$/g testis/day) | $12.87 \pm 0.29^{a}$ | $11.70 \pm 0.69^{ab}$ | $9.68 \pm 0.58^{b}$ | $11.35 \pm 0.48^{ab}$ | $11.76 \pm 0.54^{ab}$ | $12.49 \pm 0.49^{a}$ |

Values expressed as mean ± SEM, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 2.** Rate of seminiferous tubules with TUNEL-positive germ cells in relation to the total of seminiferous tubules in the same categories of stages of the seminiferous epithelium in the experimental groups on post-natal day (PND) 53.

| | Experimental Groups (n= 5) | | | | | |
|---|---|---|---|---|---|---|
| **Parameters (%)** | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Total of seminiferous tubules with TUNEL-positive germ cells | 5.00 (3.00 – 5.50) $^{a}$ | 9.00 (7.50 – 12.50) $^{b}$ | 11.00 (10.50 – 14.00) $^{b}$ | 5.00 (3.50 – 6.00) $^{a}$ | 2.00 (2.00 – 5.00) $^{a}$ | 6.00 (3.50 – 6.50) $^{a}$ |
| Stages I-VI | 6.12 (3.77 – 9.41) $^{a}$ | 13.51 (10.71 – 16.60) $^{b}$ | 13.64 (9.43 –25.88) $^{b}$ | 6.25 (4.71 – 10.32) $^{a}$ | 2.56 (2.35 – 6.98) $^{a}$ | 6.98 (6.35 – 10.53) $^{a}$ |
| Stages VII-VIII | 0 (0 – 1.61) $^{a}$ | 7.69 (0 – 15.42) $^{a}$ | 3.45 (1.67 – 9.13) $^{a}$ | 3.23 (0 – 3.58) $^{a}$ | 0 (0 – 4.63) $^{a}$ | 0 (0 – 6.25) $^{a}$ |
| Stages IX-XIII | 4.54 (2.00 – 8.35) $^{a}$ | 3.33 (0 –10.44) $^{a}$ | 12.90 (8.08 – 13.81) $^{b}$ | 2.86 (0 – 3.35) $^{a}$ | 0 (0 – 4.78) $^{a}$ | 0 (0 – 5.61) $^{a}$ |
| Stage XIV | 0 (0 – 25.00) $^{a}$ | 16.67 (0 – 32.50) $^{a}$ | 28.57 (0 – 45.00) $^{a}$ | 0 (0 – 41.67) $^{a}$ | 0 (0 – 26.67) $^{a}$ | 0 (0 – 25.00) $^{a}$ |

Values expressed as median and interquartile intervals, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 3.** Rate of seminiferous tubules with TUNEL-positive germ cells in relation to the total of the seminiferous tubules in the same categories of stages of seminiferous epithelium in the experimental groups on post-natal day (PND) 110.

| | Experimental Groups (n= 5) | | | | | |
|---|---|---|---|---|---|---|
| **Parameters (%)** | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Total of seminiferous tubules with TUNEL-positive germ cells | 6.00 (4.00 – 6.50) [a] | 11.00 (8.50 – 14.00) [b] | 14.00 (10.50 – 17.00) [b] | 7.00 (5.00 – 7.00) [a] | 8.00 (7.00 – 8.00) [a] | 8.00 (6.00 – 8.00) [a] |
| Stages I-VI | 6.98 (4.82 – 12.20) [a] | 13.51 (10.71 – 16.60) [b] | 13.64 (9.43 –25.88) [b] | 6.25 (4.71 – 10.32) [a] | 2.56 (2.35 – 6.98) [a] | 6.98 (6.35 – 10.53) [a] |
| Stages VII-VIII | 0 (0 – 0) [a] | 0 (0 – 1.72) [a] | 0 (0 – 0) [a] | 0 (0 – 0) [a] | 0 (0 – 3.57) [a] | 0 (0 – 6.98) [a] |
| Stages IX-XIII | 4.76 (2.27 – 11.26) [a] | 10.81 (9.09 –13.39) [ab] | 17.86 (8.70 – 21.06) [b] | 7.69 (3.57 – 11.14) [a] | 12.50 (7.63 – 15.14) [ab] | 6.67 (4.38 – 12.70) [a] |
| Stage XIV | 0 (0 – 21.43) [a] | 33.33 (14.29 – 54.76) [a] | 50.00 (20.00 – 65.00) [b] | 25.00 (0 – 36.67) [a] | 25.00 (7.14 – 41.67) [a] | 0 (0 – 30.00) [a] |

Values expressed as median and interquartile intervals, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 4.** Rate of seminiferous tubules in each stage of the cycle of the seminiferous epithelium in the experimental groups on PND 110.

| | Experimental Groups (n= 7) | | | | | |
|---|---|---|---|---|---|---|
| **Parameters (%)** | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Stage I | 9.50 (8.75 – 12.50) | 10.00 (7.75 – 11.25) | 10.00 (8.25 –11.00) | 11.00 (8.25 –12.50) | 12.50 (11.75 – 13.50) | 12.00 (11.00 – 12.25) |
| Stage II | 6.50 (5.75 – 9.25) | 8.00 (5.75 – 9.25) | 8.00 (7.25 – 8.00) | 6.00 (5.75 – 7.25) | 6.50 (6.00 – 8.00) | 6.00 (5.00 – 7.25) |
| Stage III | 6.50 (5.00 – 8.25) | 8.00 (5.75 – 9.50) | 10.00 (5.00 – 10.25) | 10.00 (8.00 – 10.00) | 6.00 (4.75 – 9.50) | 7.00 (5.00 – 9.50) |
| Stage IV | 3.50 (3.00 – 4.00) | 3.50 (2.75 – 4.25) | 3.00 (2.75 – 5.25) | 4.00 (3.50 – 4.25) | 2.00 (2.00 – 3.50) | 3.00 (3.00 – 4.00) |
| Stage V | 5.50 (2.75 – 8.00) | 4.00 (2.50 – 5.50) | 6.50 (4.00 – 8.00) | 6.50 (4.50 – 7.50) | 4.50 (5.00 – 5.25) | 6.00 (5.00 – 7.00) |
| Stage VI | 4.50 (3.75 – 5.25) | 2.50 (2.00 – 4.00) | 3.50 (2.50 – 5.00) | 4.50 (3.75 – 5.50) | 4.00 (2.75 – 7.50) | 4.00 (3.00 – 5.25) |
| Stage VII | 20.00 (18.00 – 23.00) | 19.00 (16.25 – 20.75) | 21.00 (20.75 – 23.25) | 20.50 (19.00 – 23.00) | 18.50 (16.75 – 19.25) | 20.00 (19.50 – 21.75) |
| Stage VIII | 12.00 (10.75 – 14.25) | 10.50 (9.25 – 12.00) | 10.50 (6.75 – 11.50) | 9.50 (8.50 – 11.00) | 9.50 (7.75 – 10.75) | 8.50 (6.75 – 11.25) |
| Stage IX | 5.00 (3.25 – 7.25) | 5.00 (3.75 – 7.75) | 3.00 (3.00 – 4.75) | 6.00 (4.50 – 7.50) | 6.00 (4.75 – 7.25) | 4.50 (3.75 – 6.25) |
| Stage X | 1.00 (0.75 – 4.75) | 4.50 (2.75 – 7.00) | 2.50 (1.00 – 4.50) | 3.50 (2.00 – 5.00) | 2.50 (1.75 – 5.25) | 3.50 (1.00 – 4.25) |
| Stage XI | 4.00 (2.75 – 5.00) | 4.50 (3.00 – 6.50) | 4.00 (3.50 – 6.25) | 2.50 (2.00 – 4.25) | 3.50 (3.00 – 5.50) | 3.50 (3.00 – 5.25) |
| Stage XII | 8.00 (7.00 – 10.50) | 9.50 (9.00 – 11.00) | 10.00 (6.75 – 10.25) | 7.50 (6.00 – 8.50) | 9.50 (7.00 – 10.25) | 8.50 (7.00 – 9.25) |
| Stage XIII | 4.50 (3.25 – 6.00) | 5.50 (4.25 – 8.00) | 6.00 (4.75 – 7.25) | 5.00 (3.00 – 5.75) | 5.00 (3.50 – 7.00) | 5.00 (5.00 – 7.00) |
| Stage XIV | 5.00 (3.25 – 7.25) | 4.50 (2.75 – 7.25) | 3.00 (2.75 – 5.25) | 4.50 (3.50 – 5.25) | 4.00 (1.75 – 5.25) | 4.00 (3.50 – 4.00) |

Values expressed as median and interquartile intervals, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. .

**Figure 1.**

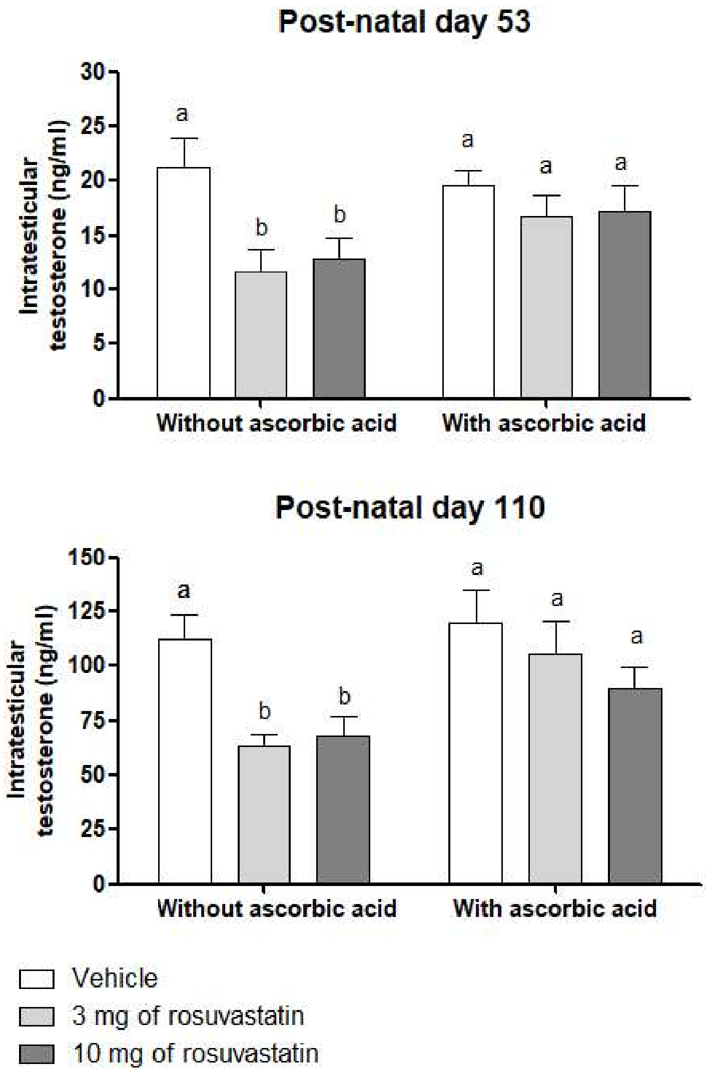

Post-natal day 53
Intratesticular testosterone (ng/ml)
0
5
10
15
20
25
30
a
b
b
a
a
a
Without ascorbic acid
With ascorbic acid
Post-natal day 110
Intratesticular testosterone (ng/ml)
0
25
50
75
100
125
150
a
b
b
a
a
a
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 2.**

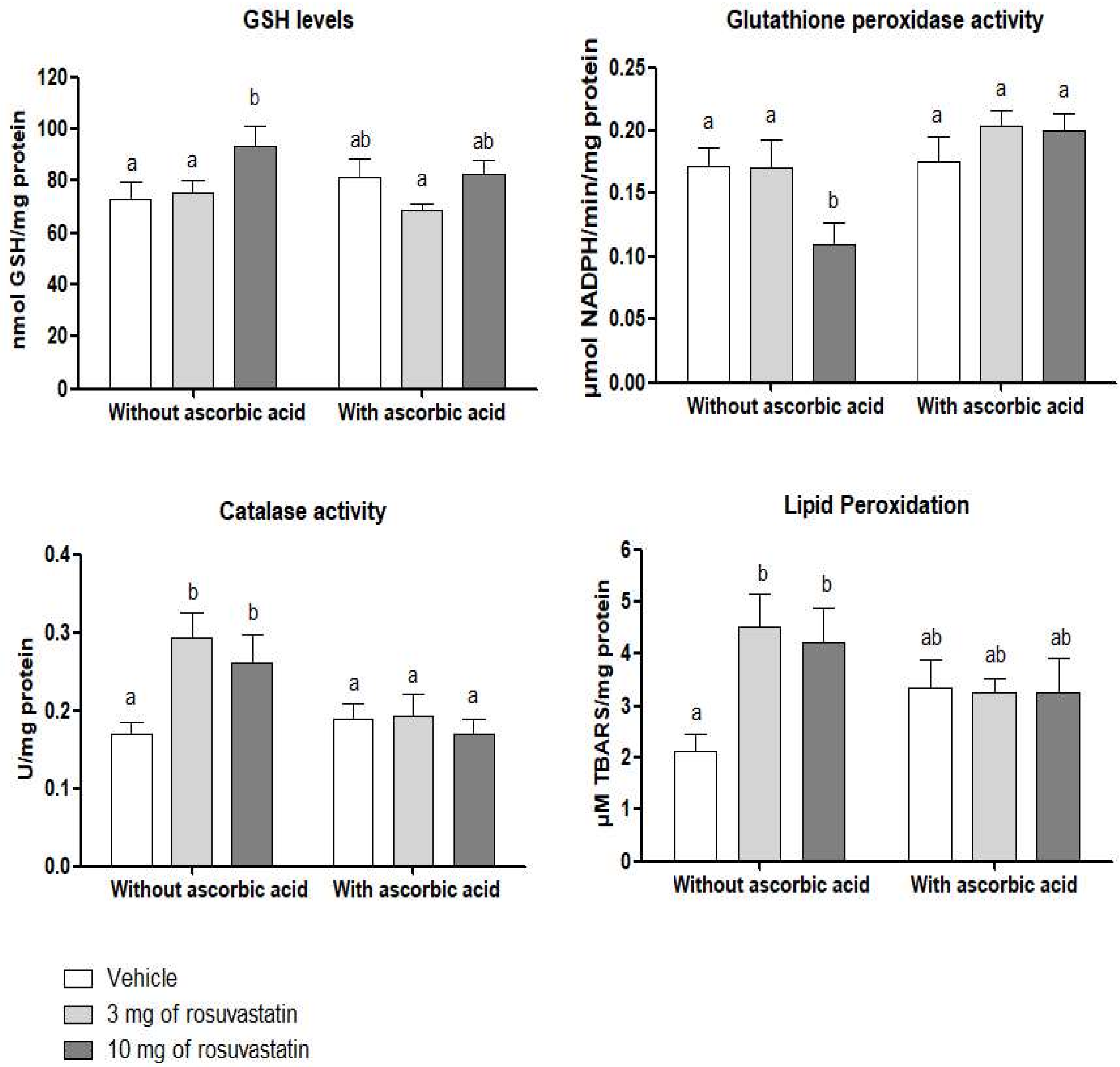

GSH levels
nmol GSH/mg protein
120
100
80
60
40
20
0
a
a
b
ab
a
ab
Without ascorbic acid
With ascorbic acid
Glutathione peroxidase activity
µmol NADPH/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
a
a
b
a
a
a
Without ascorbic acid
With ascorbic acid
Catalase activity
U/mg protein
0.4
0.3
0.2
0.1
0.0
a
b
b
a
a
a
Without ascorbic acid
With ascorbic acid
Lipid Peroxidation
µM TBARS/mg protein
6
5
4
3
2
1
0
a
b
b
ab
ab
ab
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 3.**

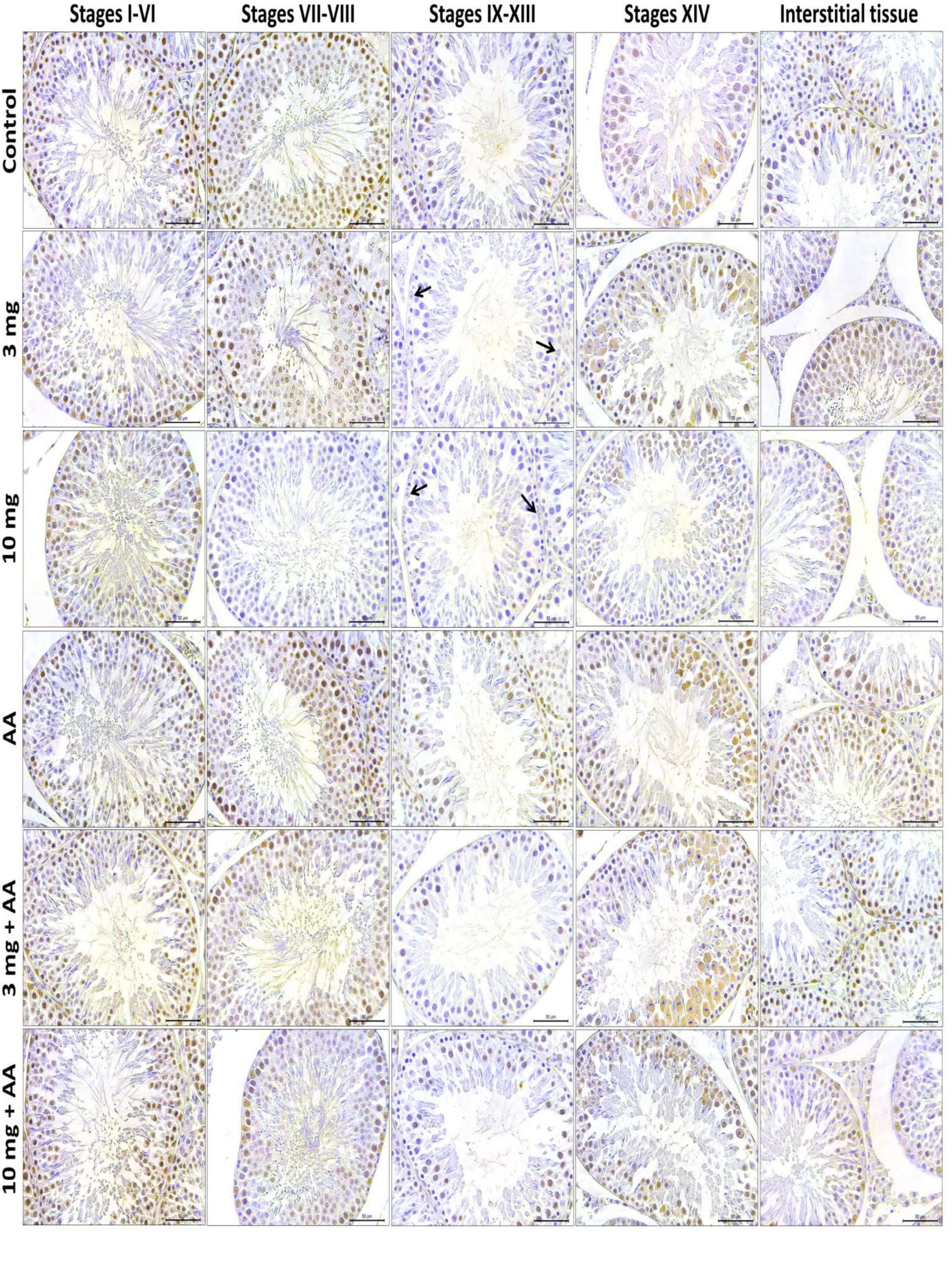

Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 4.**

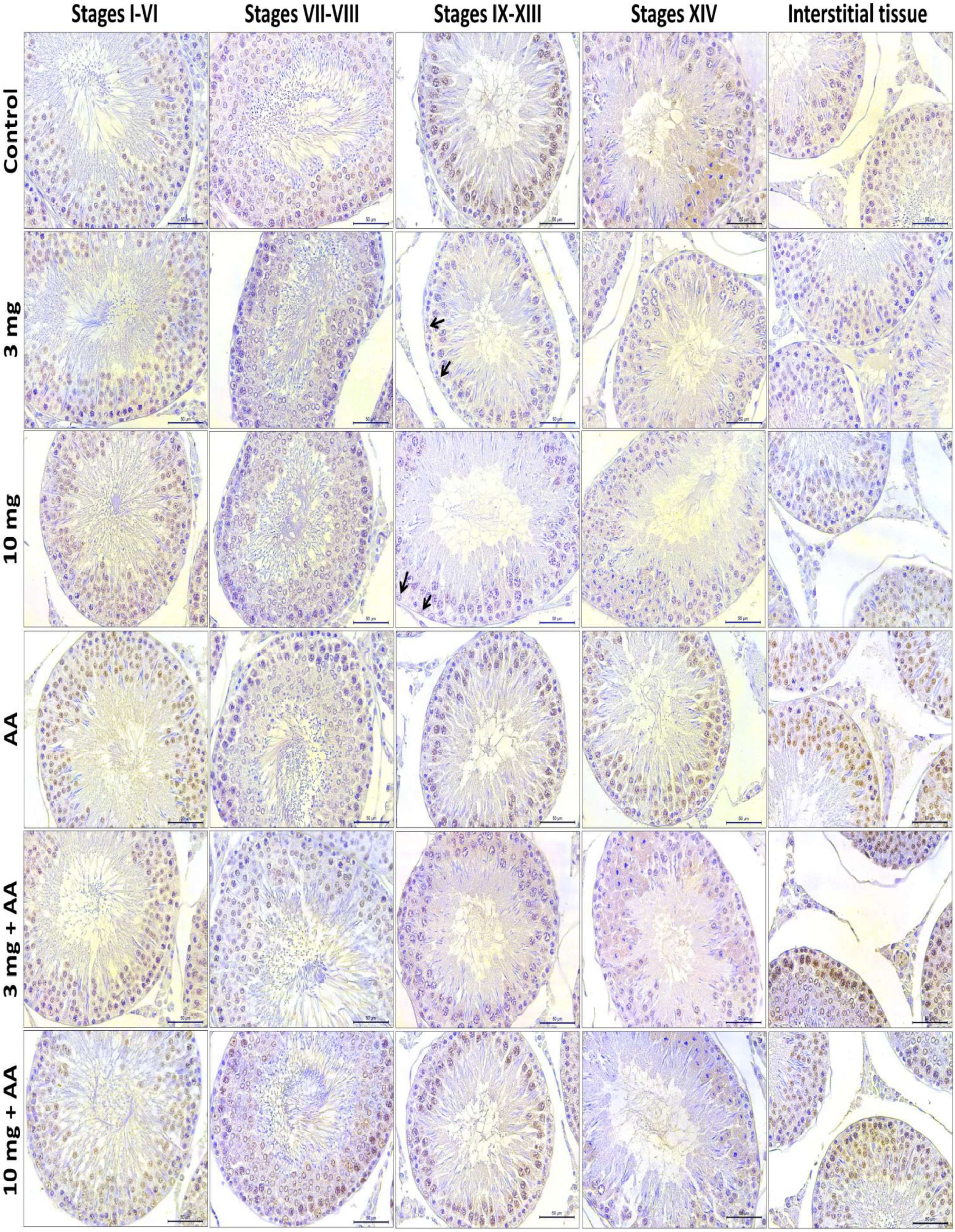

Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 5.**

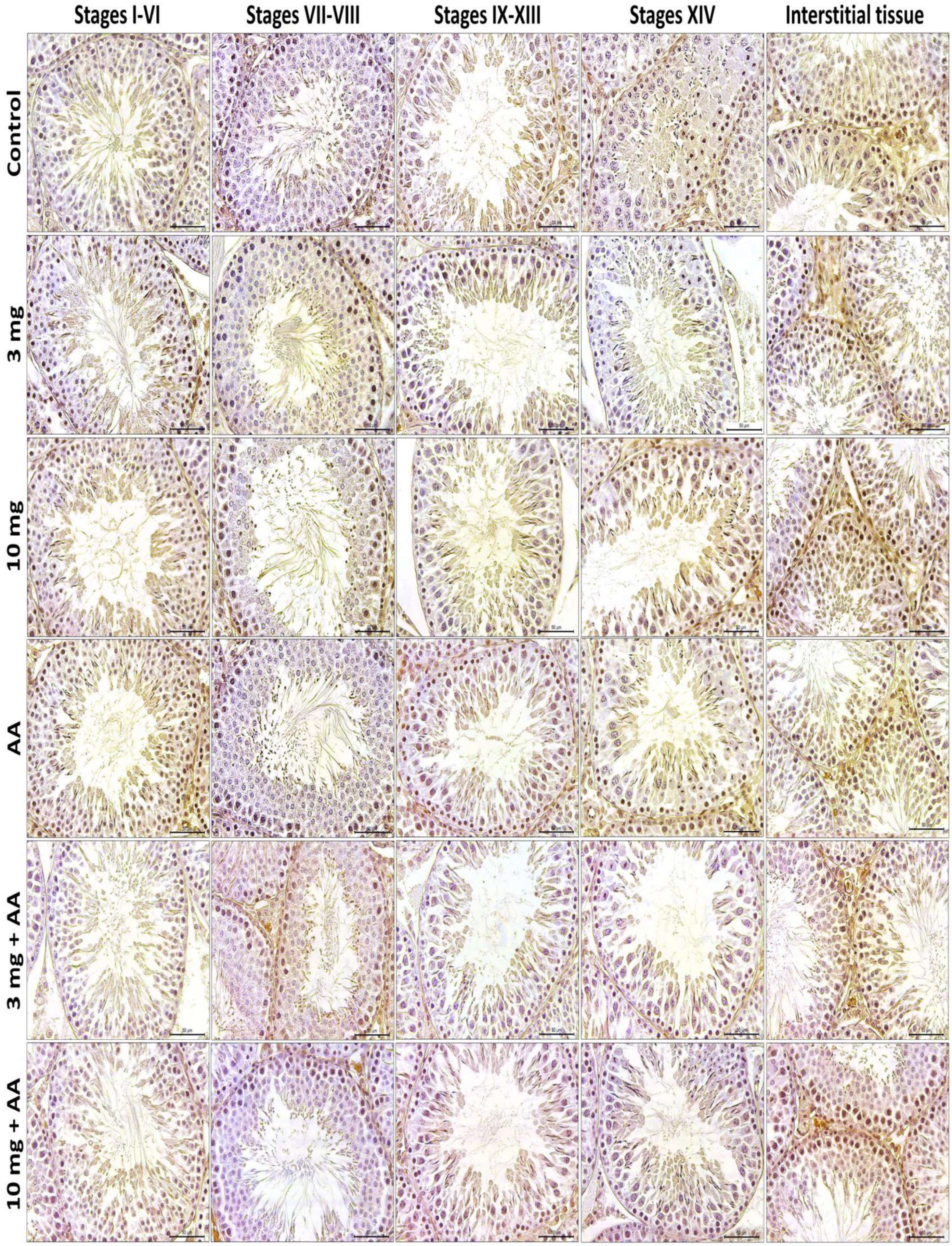
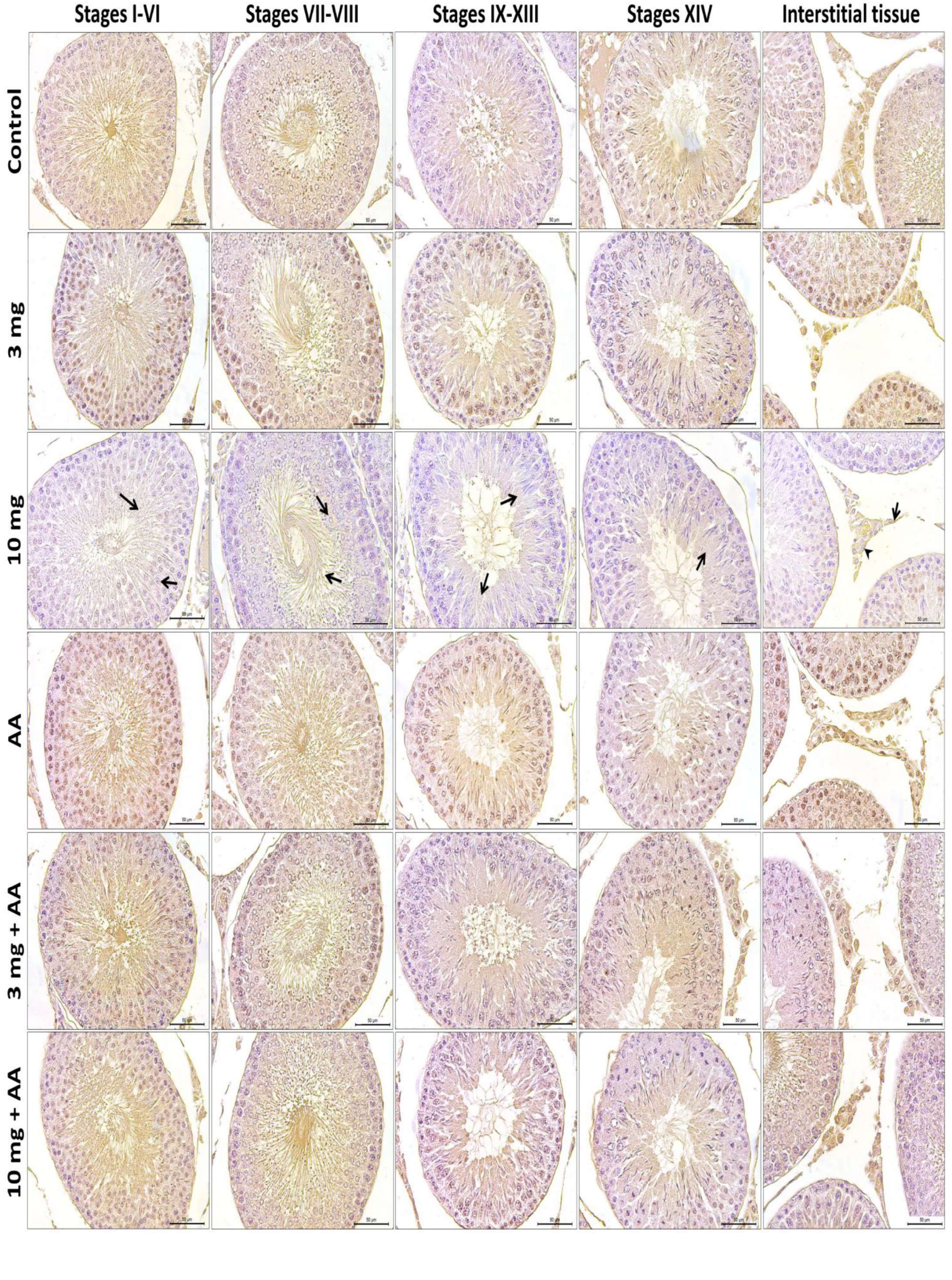
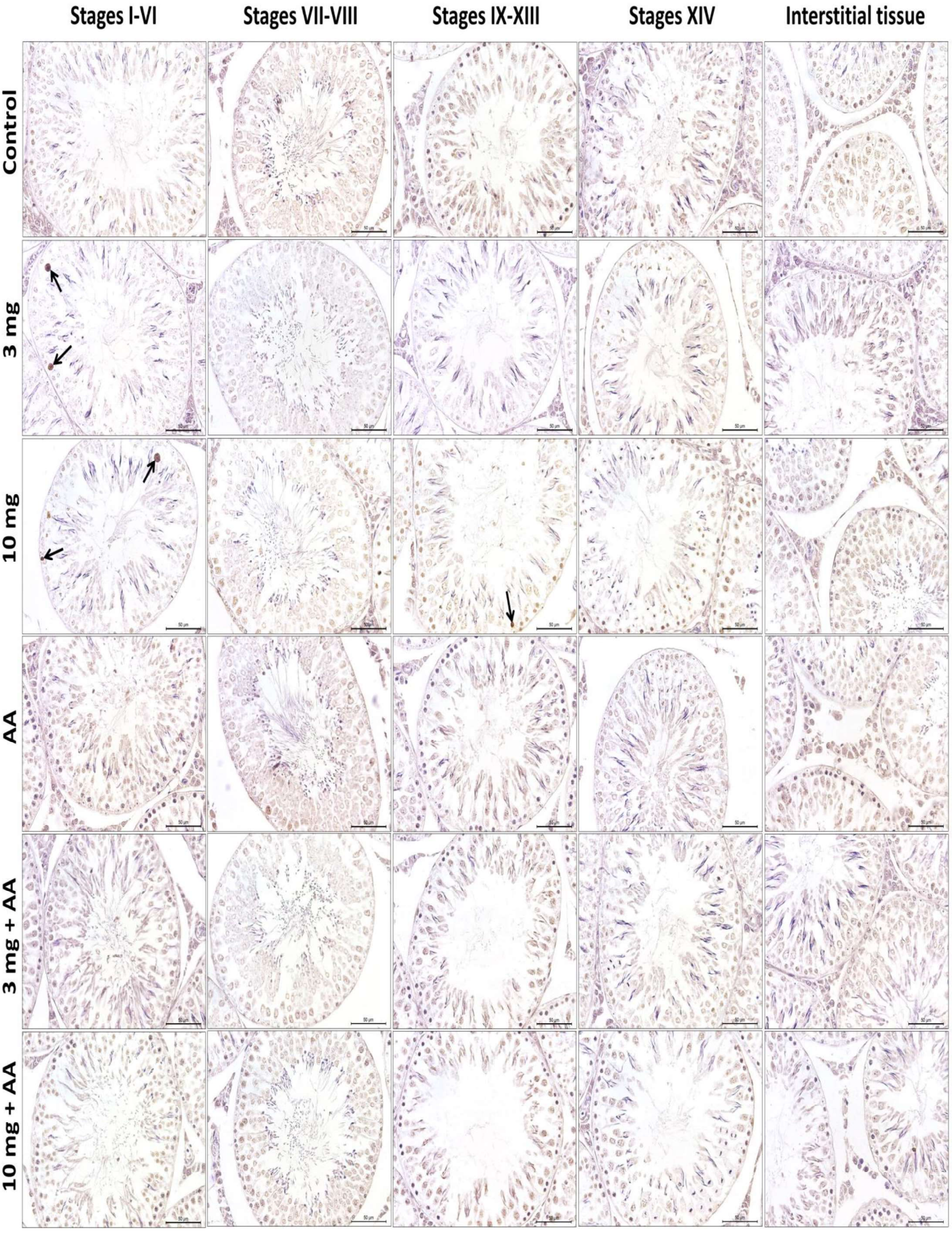

Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 6.**



Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 7.**



Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 8.**

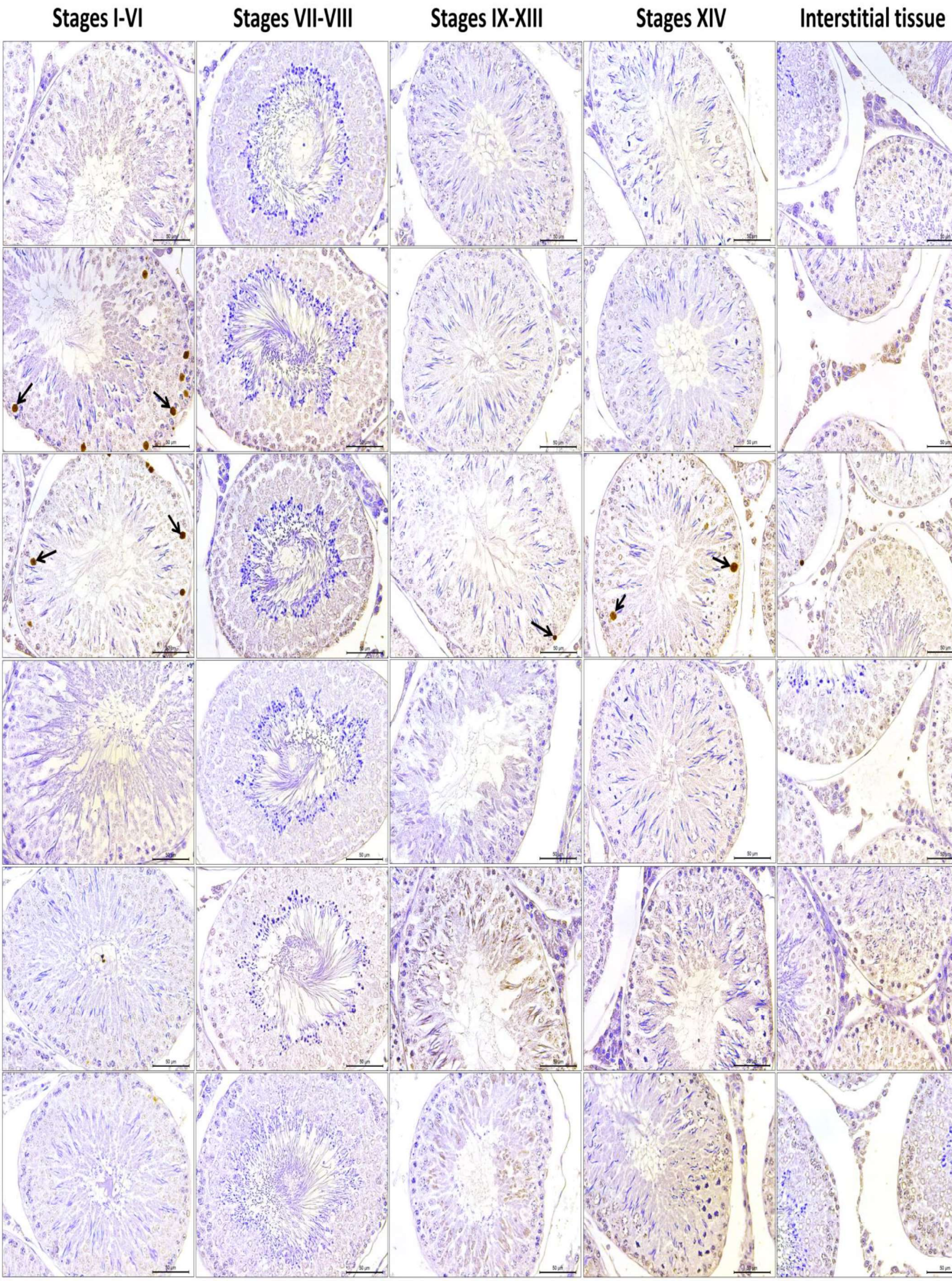

Stages I-VI
Stages VII-VIII
Stages IX-XIII
Stages XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 9.**

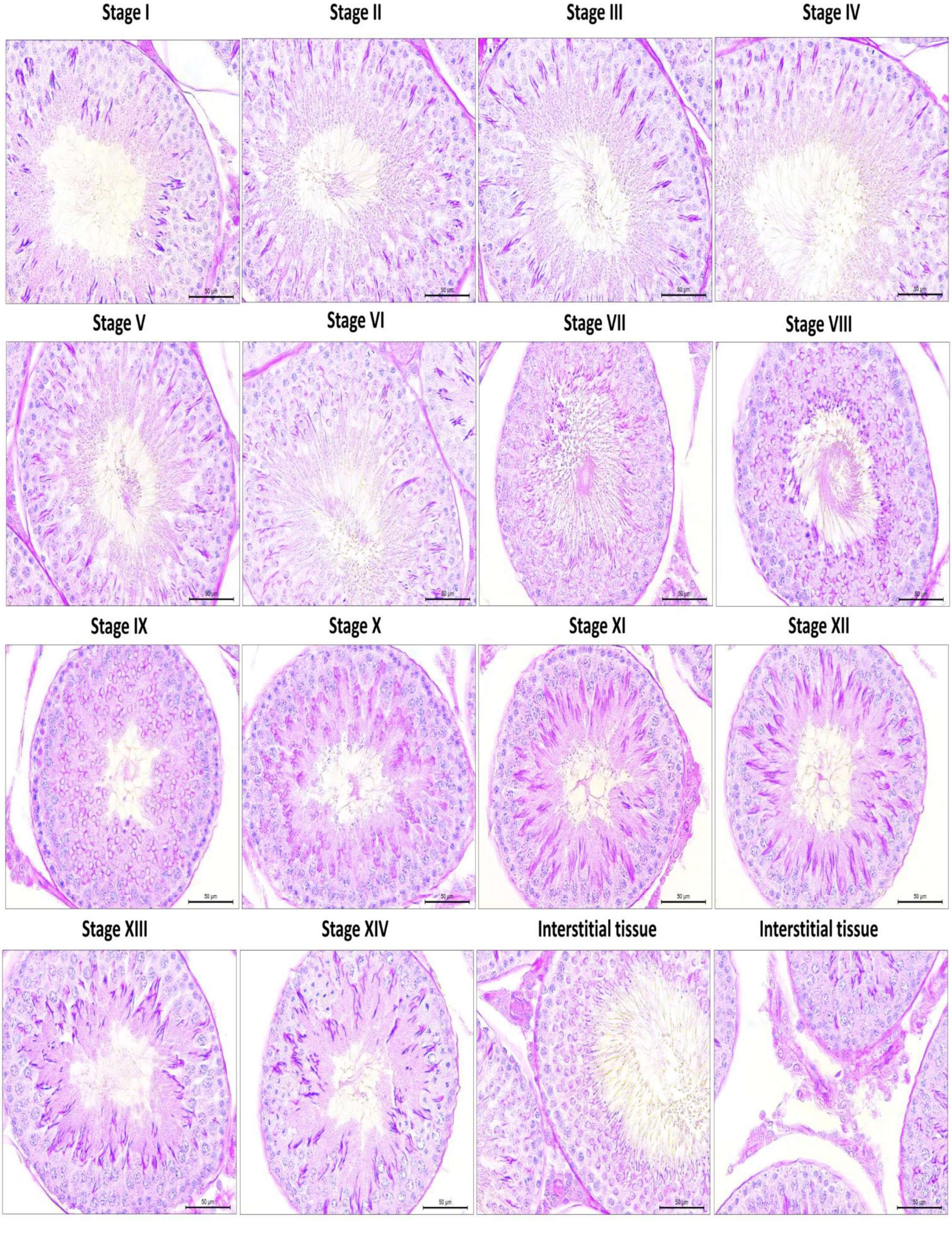
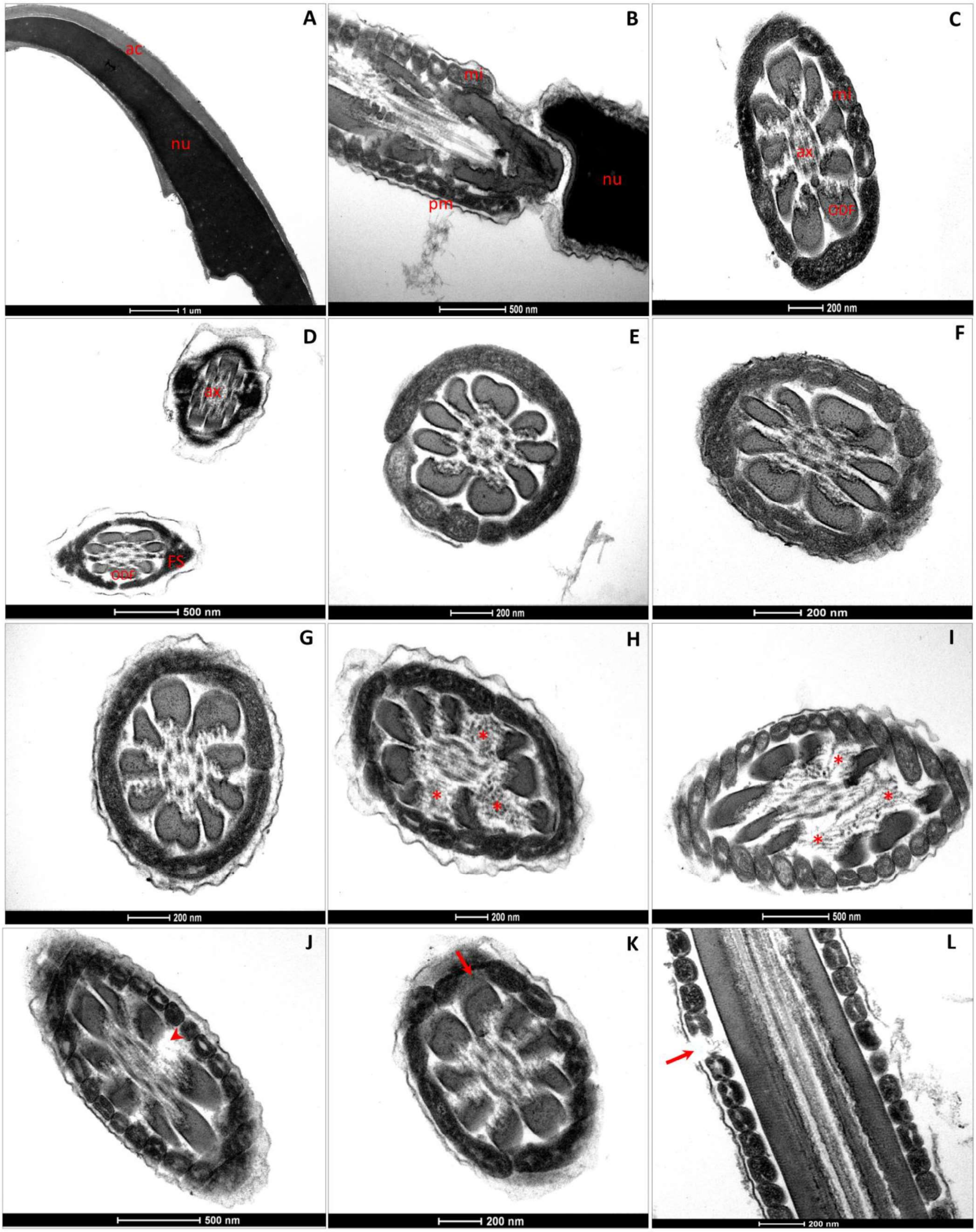
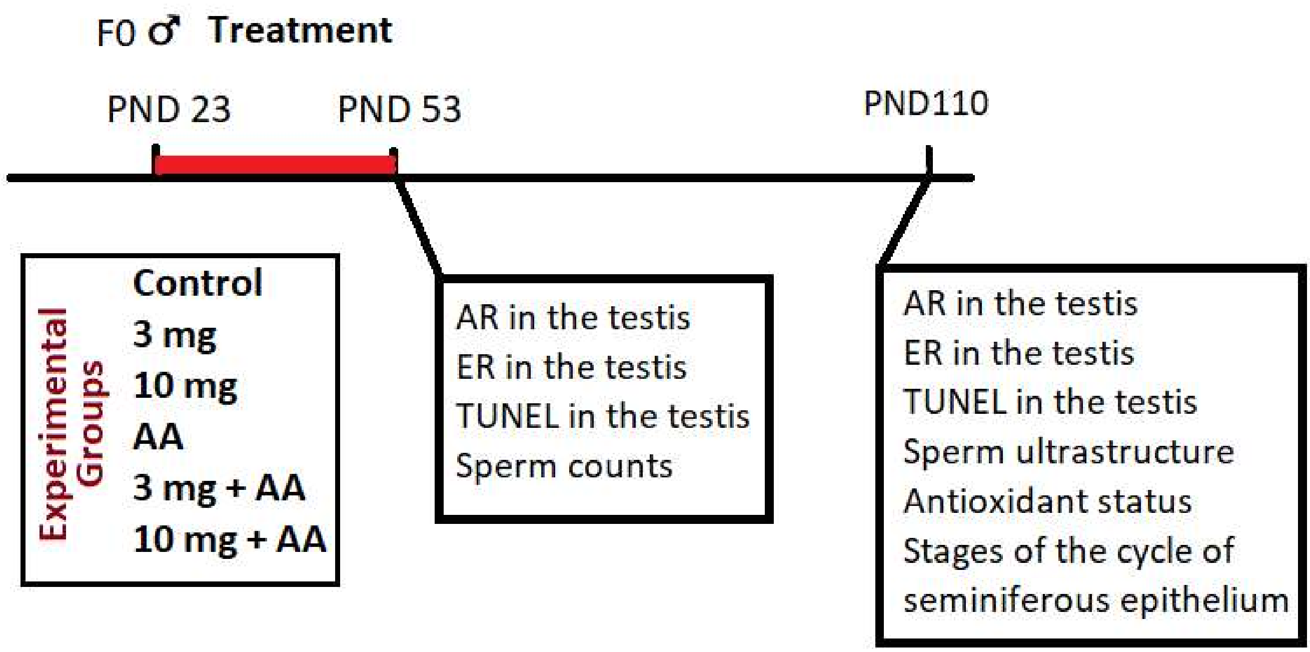

Stage I
Stage II
Stage III
Stage IV
Stage V
Stage VI
Stage VII
Stage VIII
Stage IX
Stage X
Stage XI
Stage XII
Stage XIII
Stage XIV
Interstitial tissue
Interstitial tissue
50 µm

**Figure 10.**


A
ac
nu
1 um
B
mi
nu
pm
500 nm
C
mi
ax
ODF
200 nm
D
ax
FS
ODF
500 nm
E
200 nm
F
200 nm
G
200 nm
H
200 nm
I
500 nm
J
500 nm
K
200 nm
L
200 nm

**Supplementary Material**

**Figure 1.** Description of the experimental design.



# Capítulo 4

**Manuscrito IV**

O quarto manuscrito é intitulado **“Ascorbic acid co-administered with rosuvastatin reduces reproductive impairment in the male offspring from male rats exposed to the statin at pre-puberty”** e será submetido na revista Food and Chemical Toxicology, Elsevier, ISSN: 0278-6915. Fator de impacto: 3,778.

**Ascorbic acid co-administered with rosuvastatin reduces reproductive impairment in the male offspring from male rats exposed to the statin at prepuberty**

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**Abstract**

Obesity during childhood and adolescence is closely related to dysfunctions on lipid profile and environmental factors, such as inappropriate diet and sedentary habits, which may provoke dyslipidemias in children. Rosuvastatin is a statin that decreases serum LDL-cholesterol and triglycerides levels. Ascorbic acid is an important antioxidant compound and has several roles on male reproduction. Prepubertal male rats were distributed into six experimental groups that received saline solution 0.9%, 3 or 10 mg/Kg/day of rosuvastatin, 150 mg/day of ascorbic acid, or 3 or 10 mg/Kg/day of rosuvastatin co-administered with 150 mg/day of ascorbic acid by gavage from post-natal day (PND) 23 until PND 53. Rats were maintained until sexual maturity and mated with nulliparous females to obtain the male offspring, whose animals were evaluated at adulthood in relation to sperm parameters, testicular and epididymal morphology, sexual behavior and fertility. Male offspring from rosuvastatin-exposed groups showed increased sperm DNA fragmentation, androgen depletion, augmented rate of germ cell death and impairment on epididymal structure. Furthermore, the offspring from males exposed to the higher dose of statin showed decreased sperm quality. Ascorbic acid co-administered to males ameliorated the reproductive damage in the male offspring provoked by paternal exposure to statin. In summary, paternal exposure to rosuvastatin may affect the reproduction in the male offspring; however, paternal supplementation with ascorbic acid was able to reduce the reproductive impairment in the male offspring caused by statin treatment to males.

**Keywords:** Vitamin C; statin; male offspring; reproduction; toxicology.

## 1. Introduction

Children and adolescents have been affected earlier by dysfunctions such as dyslipidemias, thus they have presented increased concentrations of serum LDL-cholesterol, VLDL-cholesterol and triglycerides, as well as diminished concentrations of HDL-cholesterol (Jiménez and Ferre, 2011; Kwiterovich, 2008a, 2008b). Currently, more attention has been paid to the pediatric population in relation to the dysfunctions associated with dyslipidemias, such as metabolic syndrome, obesity, glucose intolerance, insulin resistance and higher blood pressure (Kwiterovich, 2008b).

Pediatric dyslipidemias may have a genetic component leading to changes in lipid profile or may be related to the lack of physical exercises (Cook and Kavey, 2011; Ross, 2016), bad eating and sedentary habits (Izar et al., 2011; Ross, 2016).

Statins are the main drug class used in the treatment of dyslipidemias, thus they are lipid-lowering medications that act inhibiting the limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (Istvan and Deisenhofer, 2001; Jiménez and Ferre, 2011).

Rosuvastatin is one of the last generation statins and was approved by regulatory agencies in 2001 (McTaggart, 2003) and furthermore, it presents superior inhibitory effects on cholesterol biosynthesis and higher capacity to diminish total cholesterol concentrations (Holdgate et al., 2003; McTaggart, 2003; Vaughan and Gotto Jr, 2004).

Children and adolescents are exposed to many chemical substances and some of these chemicals may compromise puberty timing, acquisition and development of reproductive capability (Stoker et al., 2000). Considering that alterations on puberty timing may affect reproduction at adulthood, these changes supplies information about the reproductive status at sexual maturity (Mantovani and Fucic, 2014; Perobelli et al., 2013, 2012). Previous studies showed that rosuvastatin administration during pre-puberty provoked delayed puberty installation and impaired sperm quality and testicular and epididymal morphology on puberty and adulthood (Leite et al., 2017a, 2017b, 2014).

Several studies have proposed the use of substances that act as an antioxidant or present an effect on diminishing the toxic adverse effects of a medication (Corsetti et al., 2011; Fernandes et al., 2011a, 2011b; Mukhopadhyay et al., 2013; Pandir et al., 2014). Several medications are necessary for the treatment of diseases, however, they may show adverse effects during the period when people are been exposed to them (Pandir et al., 2014; Sooriyaarachchi et al., 2012).

Ascorbic acid is a vitamin needful for many biochemical reactions in the organism (Fernandes et al., 2011a, 2011b; Sönmez et al., 2005). On male reproductive system, ascorbic acid acts as an antioxidant compound against the oxidative stress and has an essential role in fertility and sperm integrity (Agarwal et al., 2005; Eskenazi et al., 2005; Fernandes et al., 2011a; Shrilatha and Muralidhara, 2007).

Moreover, various studies have shown that parental exposure to an endocrine disruptor may compromise reproduction of the exposed animals, as well as, affect reproductive function of the male or female offspring from the exposed animals even for two subsequent generations (Favareto et al., 2011; Schagdarsurengin and Steger, 2016; Silva et al., 2016; Zhao et al., 2015).

Considering the use of lipid-lowering drugs for children to improve lipid profile and to prevent cardiovascular diseases in a critical period like peri-puberty, the present study aimed to assess the reproductive parameters on male offspring from rosuvastatin-treated rats and evaluate the possible preventive role of paternal supplementation with ascorbic acid for male offspring reproduction, since previous studies reported that rosuvastatin exposure impaired reproduction of exposed animals.

## 2. Material and Methods

### 2.1. Animals

#### 2.1.1. Obtainment of pups and reduction of litters

Nonpathogenic free *Wistar* rats, both males and females with 45 days of age, were supplied from Central Biotherium of São Paulo State University (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu during the experiment.

Male and female *Wistar* rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled conditions for temperature (23 ± 1°C) and lighting conditions (12:12h photoperiod). The health condition of the animals was monitored throughout the experiment.

Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During sexual maturity two nulliparous female rats (75 days of age) were mated with one male (90 days of age) during the dark phase of the lighting cycle; the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating female rats were maintained in individual cages.

After birth, the number of pups per litter was reduced to eight on postnatal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not included in the experimental protocol and were subsequently euthanized by cervical dislocation.

**2.1.2. Experimental design**

Non-obese immature male rats were distributed into six experimental groups on PND 23 (n=10 per group, with one pup per litter for each group), that received vehicle (saline solution 0.9%, control group), supplementation with 150 mg/day of ascorbic acid (AA), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0.9% (3 mg or 10 mg) or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin (3 mg + AA or 10 mg +AA). Ascorbic acid and rosuvastatin were purchased from a commercial pharmacy (Farmácia Botica Oficinal, Botucatu/Brazil). The drugs were administered orally by gavage from PND 23 to PND 53, following male pubertal assay of 31 days recommended by U.S. Environmental Protection Agency (EPA) (Stoker et al., 2000). This period of treatment represents a critical period for development and maturation of reproductive system (Leite et al., 2017b; Stoker et al., 2000).

The doses of rosuvastatin between 5 and 40 mg/day are frequently used by humans to decrease total cholesterol and LDL-cholesterol (Vaughan **&** Gotto Jr, 2004), thus the doses used in this study were based on body surface area correction from children available doses of rosuvastatin to prepubertal rats equivalent doses (Reagan-Shaw et al., 2008). The doses of ascorbic acid supplementation were based on previous studies (Fernandes et al., 2011a, 2011b).

Male rats (generation F0) were maintained until postnatal day (PND) 110 when were mated with nulliparous female rats to obtain their male offspring (generation F1). After the birth of pups, litters were reduced to eight pups on PND 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not included in the experiment and thereafter, were euthanized. Males from generation F0 were used for a previous study (Leite et al., 2017a)

Male offspring were maintained until PND 110, after performing the sexual behavior test on PND 100, in order to evaluate the intergenerational reproductive effects due to paternal exposure to rosuvastatin and/or ascorbic acid supplementation during pre-puberty. Thereby, the following parameters were obtained: final body weight, reproductive and vital organ weights, testicular and epididymal histopathology and spermatogenesis kinetics, as wells as, sperm counts, motility and morphology, sperm DNA damage and hormonal concentrations. Furthermore, male rats were assessed in relation to their reproductive performance and fertility after sexual behavior test.

Rats were monitored in relation to the indications of distress, such as the presence of bristling hair and the ingestion of food and water during the whole experiment. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

**2.2. Evaluation of male sexual behavior**

Male rats were placed individually in polycarbonate crystal cages, measuring 44 x 31 x 16 cm, five minutes before the introduction of one sexually receptive nulliparous adult female in natural proestrus or estrus determined by vaginal smear. Behavioral testing was assessed in the dark phase of the cycle between 8:00 am and 12:00 pm in a separate room under dim red illumination. If the male rat did not mount within the next 10 minutes, a second try was allowed on the next day.

The following measures were recorded (Ahlenius & Larsson, 1984): intromission latency, defined as the time from introduction of the female in the cage to the first intromission; the number of mounts and intromissions preceding the first ejaculation; ejaculation latency, the time from introduction of the female in the cage to the first ejaculation; latency to the first post-ejaculatory intromission, the time to the first intromission after the first ejaculation; the number of intromissions after the first ejaculation and total number of ejaculations. During the second chance, if the male did not mount in the first 10 minutes following the introduction of one receptive adult female into the cage, it was considered sexually inactive.

**2.3. Euthanasia of the rats, body weight and organ weights**

Male offspring (one male per litter) were weighed on PND 110 and euthanized following narcosis by $CO_2$ asphyxiation and thereafter, blood was collected by inferior vena cava, between 9:00 and 11:30 a.m. Reproductive organs, such as left testis, epididymis and vas deferens, seminal gland (full and empty, without the coagulating gland) and ventral prostate, from the rats were collected and weighed. Vital organs that have an important role for toxicological parameters, such as kidneys, adrenal glands, liver, thyroid, pituitary and brain were also obtained and weighed.

**2.4. Hormonal concentrations**

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal dosages. Furthermore, testicular fluid was obtained by centrifugation (10000 x g, 5 minutes, 4ºC) of the right testis (without tunica albuginea) in a refrigerated device and was frozen at – 80ºC until the moment of hormonal determination. Testosterone, FSH and LH were determined by the double-antibody radioimmunoassay. Plasma LH and FSH concentrations were determined using specific kits provided by the National Hormone and Peptide Program (Harbor-UCLA, USA). The primary antibodies for LH and FSH were anti-rat LH-S10 and FSH-S11 and the references were LH-RP3 and FSH-RP2, respectively. The lower limit of detection for LH was 0.04 ng/mL and for FSH, 0.2 ng/mL. The intra-assay coefficients of variation were 3.4% for LH and 3.0% for FSH. Plasma levels of testosterone were determined using specific kits provided by MP Biomedicals (Orangeburgh, NY, USA). The lower limit of detection was 0.07 pg/mL and the intra-assay coefficient of variation was 4.0%. All samples were measured in duplicates and included in the same assay to avoid inter-assay errors.

**2.5. Sperm motility**

Sperm motility was assessed soon after euthanasia. The right vas deferens was collected, sperm were obtained and a sample was diluted in 2 mL of the modified HTF medium (Spectrun 90126), then a 10 μL aliquot was transferred to a Mackler chamber. Under a light microscope (20x magnification), 100 spermatozoa were analyzed and classified as: type A, motile with regular and fast progressive movement; type B, motile with non-progressive movement or type C, immotile. Sperm motility was expressed as the percentage of total sperm (Perobelli et al., 2012).

## 2.6. Sperm morphology

Sperm were obtained from the right vas deferens of the male rats from the generation F1, diluted in modified HTF medium and a sample was obtained and added to 1.0 mL of saline formol. For the analysis, smears were prepared on histological slides that were left to dry for 90 minutes and observed in a phase-contrast microscope (400X magnification); 200 spermatozoa were analyzed per animal (Seed et al., 1996). Morphological abnormalities were classified into general categories pertaining to head morphology (without curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken, isolated, i.e., no head attached, or rolled into a spiral). Moreover, the presence and position (proximal, medial or distal) of the cytoplasmic droplet were evaluated in the same sperm (Filler, 1993).

## 2.7. Sperm counts in the testis and epididymis and sperm transit time

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were enumerated as previously reported (Robb et al., 1978), with the following adaptations: the right testis from one male rat of each litter were decapsulated and weighed immediately after collection, were homogenized in 5 mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution, a sample was transferred to Neubauer chambers (4 fields per animal), where mature spermatids were enumerated. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium.

To obtain the number of mature spermatids per gram of testis and the relative DSP, the number of mature spermatids and the DSP were divided by the weight of the testicular parenchyma. In the same manner, caput/corpus and cauda epididymis parts were cut into small fragments with scissors and homogenized, and sperm enumerated as described for the testis. The sperm transit time throughout the epididymis was determined by dividing the number of sperm in each part of the organ by the DSP.

**2.8. Sperm DNA damage (Comet assay)**

The comet assay was used for detecting primary DNA damage (single- and double-strand breaks and alkali-labile sites) in sperm isolated from the cauda epididymis that were maintained in modified HTF medium stored at -80°C. The alkaline version of the assay was carried according to a previous study (Tice et al., 2000). Briefly, the samples were thawed and 5 µL of the HTF medium with sperm sample was mixed with 75 µL of low-melting-point agarose (0.5%) and then placed onto slides that had previously been covered with a thin layer of normal-melting-point agarose (1.5 %). The slides were covered with lysis solution (100mM $Na_2$-EDTA, 10mM Tris-HCl, 2,5M NaCl, pH 11, containing 40mM DTT and 2% Triton X-100) and incubated for 1 hour at 4 °C. Thereafter, the slides were submitted to a second lysis solution containing proteinase K (100 µg/ml) for 2 hours and 30 min at 37 °C and were subsequently incubated in a horizontal electrophoresis tank containing freshly prepared cold alkaline electrophoresis buffer (300mM NaOH, 1mM Na2 EDTA, pH > 13) for 45 min to allow the DNA to unwind and for alkali-labile site expression in an air conditioned room (18 ºC) . Electrophoresis was conducted in the same alkaline buffer for 20 min at 3 V/cm and 270 mA at 4 °C. Then, the slides were washed in PBS solution, rinsed in water, fixed in absolute ethanol and stored at room temperature until analysis. The slides were stained with SYBR® Gold (1:10,000; Invitrogen; Grand Island, NY, USA) immediately before analysis. A total of 100 randomly selected nucleoids per animal was analyzed under 400× magnification with a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill, UK). Tail intensity (% DNA in the tail) was used to estimate DNA damage. The slides were prepared in duplicate and all steps were conducted in the dark to prevent additional DNA damage.

**2.9. Histological procedures**

The left testis and epididymis from one male per litter were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 µm cuts (transversal sections of the testis and longitudinal sections of epididymis). Sections were stained with hematoxylin and eosin (HE) to evaluate testicular and epididymal histology under light microscope. The evaluation was conducted in a blind assay and the photomicrographs were obtained using a Leica light microscopy, coupled to a digital camera and a personal computer with the software Leica Qwin Version 3 for Windows.

Seminiferous tubule cross-sections were randomly chosen in three non-serial testicular sections per animal obtained with a distance of 50 µm among them, totaling 200 tubules evaluated per animal. Seminiferous tubules were classified as: normal (presence of concentric and normally organized germ cell layers in seminiferous epithelium) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, seminiferous epithelium with acidophilic cells, few germ cell layers, vacuole formation or degeneration in seminiferous epithelium).

Histopathological evaluation of interstitial tissue and peritubular myoid cells was qualitative; the interstitial analysis aimed to assess Leydig cells morphology and the appearance of blood vessels. The epididymal histopathological analysis was also qualitative to evaluate each region of the organ according to the epithelium, lumen and interstitial tissue morphology.

**2.10. Spermatogenesis kinetics**

To evaluate spermatogenesis kinetics, one hundred random tubular sections per animal (n = 9 or 10 animals⁄ group) in three non-consecutive testis cross-sections were classified into four categories: stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelium cycle (Leblond **&** Clermont, 1952), under a light microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at x200 magnification.

**2.11. TUNEL assay**

For evaluation of *in situ* DNA fragmentation associated with cell death, TUNEL assay was performed using a commercial kit (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit S7100, Chemicon, Temecula, CA). The manufacturer's instructions were properly followed. Testicular sections from adult male rats were sectioned in 4 µm cuts, were allocated in silanized slides, dewaxed using xylene (Sigma-Aldrich®, USA), hydrated using decreasing concentrations of ethanol and were washed in PBS buffer. Sections were incubated with proteinase K (20 µg/mL) for 15 minutes, washed with PBS buffer and then, endogenous peroxidase was quenched with 3% of hydrogen peroxide in PBS buffer for 5 minutes. After washing, the sections were exposed to equilibration buffer followed by incubation with TdT enzyme for 1 hour in an incubator at 37°C. The reaction was blocked with stop buffer, followed by washing of the sections with PBS buffer and incubation with anti-digoxigenin-peroxidase for 30 minutes at room temperature. The slides were washed with PBS buffer, and then, submitted to diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich®, MO, USA) for 3 minutes. After this step, testicular sections were counterstained with hematoxylin, dehydrated in crescent concentrations of ethanol and immersed in xylene. Coverslips were mounted on the slides with Entellan. Negative controls were performed without TdT enzyme. Testicular sections were evaluated in the relation of presence of marked cells in different categories of stages of seminiferous epithelium (stages I-VI, VII-VIII, IX-XIII and XIV) and the results were expressed as a percentage of seminiferous tubules with TUNEL-marked germ cells in each category of stages. All analyses were carried out using a Leica light microscope, coupled to a digital camera and a personal computer with the software Leica Qwin Version 3 for Windows.

**2.12. Fertility and reproductive performance**

This analysis was performed following natural mating. In the case of rats that ejaculated during the evaluation of sexual behavior, couples stayed together for an additional four hours, allowing a greater number of ejaculations. Animals that had been deemed inactive were tested again daily for the next 5 consecutive days during which different receptive females were placed in their boxes during the dark phase of the cycle.

Every morning, males considered inactive on sexual behavior testing were separated from the females, and vaginal smears of each female were examined for the presence of sperm. The day on which sperm were found in the vaginal smear was considered day 0 of gestation (GD 0). On the 20th day of gestation (GD 20), females were weighted and afterward euthanized following narcosis by $CO_2$ asphyxiation and decapitation. After collection of the uterus and ovaries, gravid uterus weight was obtained; corpora lutea, implantation sites, reabsorptions and live fetuses were enumerated and fetal and placental weights were determined.

The following rates were determined based on these results: gestation rate = number of pregnant females/ number of inseminated females x 100; fertility potential (efficiency of implantation) = implantation sites/ corpora lutea x 100; rate of post-implantation loss = number of implantations - number of live fetuses/ number of implantations x 100; sex ratio = number of male fetuses/ number of female fetuses x 100.

### 2.13. Statistical analysis

The results from the different parameters were compared and analyzed among the experimental groups using two-way ANOVA, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were carried out on GraphPad Prism (version 5.00).

## 3. Results

### 3.1. Testicular and epididymal morphology and spermatogenesis kinetics

Male offspring from rosuvastatin-treated groups showed lower percentage of normal seminiferous tubules in the testis, followed by an increased rate of acidophilic germ cells in the seminiferous epithelium when compared to the control group ($p < 0.05$) (Table 1 and Figure 1). Paternal supplementation with ascorbic acid prevented the diminished rate of normal seminiferous tubules and the avoided the raised percentage of acidophilic germ cells in the seminiferous epithelium of the progeny (Table 1 and Figure 1). Spermatogenesis kinetics evaluated using categories of seminiferous epithelium stages showed no differences among the male offspring of the experimental groups (Table 1).

The experimental groups whose fathers were exposed to both doses of rosuvastatin showed an increased rate of TUNEL-positive germ cells, spermatogonias and spermatocytes, in the seminiferous epithelium on stages I-VI and IX-XIV ($p < 0.05$) (Table 2 and Figure 2). The male offspring from ascorbic-acid-supplemented groups exhibited a rate of seminiferous tubules with TUNEL-marked germ cells similar to the controls (Table 2 and Figure 2).

Epididymal morphology from the progeny of the rosuvastatin-exposed groups showed apparent hyperplasia of clear cells at proximal cauda of the organ, in comparison with control group (Figure 3). On the other hand, paternal supplementation with ascorbic acid prevented this histopathological alteration on male offspring (Figure 3).

### 3.2. Sperm parameters

Male offspring from the group exposed to the higher dose of rosuvastatin presented lower daily sperm production and a diminished number of mature spermatids in the testis at adulthood, when compared to the control group ($p < 0.05$) (Table 3). Moreover, the progeny of rosuvastatin-treated groups showed lower relative daily sperm production and reduced number of mature spermatids per gram of testis in a dose-dependent manner ($p < 0.05$) (Table 3). Ascorbic acid supplementation for males on generation F0 was able to prevent the decreased sperm production per testis in the offspring (Table 3).

Relative sperm number in the epididymis caput/corpus and cauda was reduced in the male offspring from the group treated with the higher dose of statin, in comparison with the controls ($p < 0.05$) (Table 3). Paternal supplementation with ascorbic acid might avoid this reduced sperm storage in the epididymis of the male offspring from co-exposed groups (Table 3). Sperm transit time throughout the epididymis caput/corpus and cauda was similar among the groups, as well as, total sperm transit time (Table 3).

Sperm motility was lower in the group whose fathers were exposed to 10 mg of rosuvastatin, exhibiting a diminished percentage of progressive and non-progressive sperm and increased frequency of immotile sperm when compared to the control group ($p < 0.05$) (Figure 4). Ascorbic acid administered to the fathers partially improved the sperm motility on male progeny from co-exposed groups (Figure 4). On the other hand, the percentage of normally shaped sperm and sperm head and tail abnormalities were similar among the experimental groups (Table 4).

Sperm DNA damage was increased in a dose-dependent manner in the male offspring from statin-treated groups, in comparison with the control group ($p < 0.05$) (Figure 5). Paternal supplementation with ascorbic acid prevented the increased sperm DNA damage in the male offspring from co-exposed groups (Figure 5).

### 3.3. Hormonal concentrations, sexual behavior and reproductive performance

Serum and intratesticular testosterone showed diminished concentrations on male offspring from rosuvastatin-treated groups in a dose-dependent manner when compared to the controls ($p < 0.05$) (Figure 6). LH concentrations were increased in the group whose fathers were exposed to the higher dose of statin, in comparison with controls ($p < 0.05$) (Figure 6). Paternal supplementation with ascorbic acid prevented the diminished testosterone concentrations and avoided the increased LH concentrations on male progeny (Figure 6). FSH concentrations were similar among the offspring of the experimental groups (Figure 6).

Sexual behavior was similar among the groups for all the parameters evaluated in this study ($p > 0.05$) (Table 5). Reproductive performance and fertility of the males in the generation

F1, assessed by natural mating, were not affected by paternal exposure to the statin and/or ascorbic acid supplementation ($p > 0.05$) (Table 6).

### 3.4. Body weight and organ weights

Final body weight did not exhibit significant differences among the male offspring of the experimental groups ($p > 0.05$) (Table 7). Reproductive organs assessed in this study, such as testis, epididymis, vas deferens, prostate and seminal gland showed similar weights among the groups ($p > 0.05$) (Table 7). In addition, vital organ weights evaluated, such as pituitary, thyroid, liver, adrenal glands, kidneys and brain, also presented no differences among the male offspring of the experimental groups ($p > 0.05$) (Table 8).

## 4. Discussion

Statins have impaired male reproduction during puberty and on sexual maturity (Leite et al., 2017a, 2017b, 2014); however they are recommended to be used by children and adolescents as lipid-lowering drugs to diminish serum total cholesterol levels. Ascorbic acid has shown a protective role in male reproduction against toxic effects provoked by statin exposure (Leite et al., 2017a, 2017b). The present experimental study aimed to assess the intergenerational reproductive effects in the male offspring of male rats that were exposed to rosuvastatin and/or ascorbic acid during pre-puberty.

This study was performed using non-obese immature Wistar rats; however, it simulates the human situation, since previous studies have reported that pediatric population has exhibited increased frequency of atherosclerosis and also needs to use statins to treat the disease (Ross, 2016; Wiegman et al., 2015); Additionally, more people are taking benefits from the pleiotropic effects of statins (Ludman et al., 2009; Tandon et al., 2005). Furthermore, there is a lack of information about the effects of paternal exposure to rosuvastatin and/or ascorbic acid and the reproductive effects for male offspring.

Androgens are necessary for various biological functions on body, such as the development, maturation and maintenance of male reproductive system, acquisition of secondary sexual characteristics and body growth (Adamopoulos et al., 1990; Mantovani and Fucic, 2014; Marty et al., 2003; Pihlajamaa et al., 2015).

In the present study it was shown that serum and intra-testicular testosterone levels of male offspring from rosuvastatin exposed groups were decreased a dose-dependent manner in comparison to control group. Since LH levels showed the opposite profile. i.e., a tendency to increase with 3 mg, and a significant increase with 10 mg of the statin, the data suggest that the primary site affected by the statin was the testis, seeing that decreases in testosterone secretion by the testis result in decreased negative feedback in pituitary gonadotrophs thus increasing the release of LH. In fact, testosterone produced by Leydig cells is the main modulator of LH secretion while FSH secretion is negatively controlled by inhibin produced by Sertoli cells. Although we did not measure inhibin, we might suggest that the inhibin secretion was not significantly affected by the statin since FSH levels was not modified by any of the statin doses.

The reduced androgens concentrations may be the cause for the reproductive impairment. It was shown that this hormonal deficiency is related to delayed epididymal differentiation, increased rate of germ cell death in the testis (Leite et al., 2017b, 2014) and augmented sperm DNA damage (Leite et al., 2017a).

On testis, Sertoli cells need testosterone to bind their androgen receptors to synthetize proteins related to the survival of germ cells (De Gendt et al., 2004; Stanton et al., 2012). Additionally, when there is testosterone depletion, germ cells undergo cell death (De Gendt et al., 2004; Stanton et al., 2012). Male offspring from rosuvastatin-treated rats, at both doses, showed increased frequency of acidophilic germ cells and TUNEL-positive germ cells on seminiferous epithelium during sexual maturity that represents augmented rate of germ cells undergoing death. On the other hand, male offspring from ascorbic-acid-supplemented groups did not exhibit this augmented rate of germ cells undergoing death, suggesting that paternal supplementation with ascorbic acid was able to prevent this impairment in the offspring. However, spermatogenesis kinetics were similar among the offspring of the experimental groups.

Previous studies reported that prepubertal exposure to rosuvastatin provoked delayed epididymal differentiation (Leite et al., 2017b, 2014) and consequently hyperplasia of clear cells on epididymis cauda at adulthood (Leite et al., 2017a). Clear cells have an important role on the reabsorption of sperm cytoplasmic droplets (Hermo et al., 1992). Furthermore, clear cells are associated with epididymal spermiophagy under normal condition (Kempinas and Klinefelter, 2015), thus increased hyperplasia of clear cells may be related to increased sperm phagocytosis in the epididymis. Epididymal morphology of male offspring from rosuvastatin-exposed rats showed hyperplasia of clear cells on proximal cauda. The groups whose fathers received ascorbic acid did not show this histopathological alteration. The remaining regions of the organ, considering the epithelium, lumen and interstitium showed to be similar among the groups.

Sperm evaluations such as sperm counts, morphology and motility have been used in association as an indicative of sperm quality (Perreault and Cancel, 2001). Lower sperm counts have been associated with diminished male fertility and it is considered as a toxic effect for male reproduction (Perreault & Cancel, 2001). Sperm morphology is used as a parameter to infer the damage that occurred during spermatogenesis on seminiferous epithelium after exposure to a toxicant (Filler, 1993). Furthermore, decreased sperm motility is associated with alterations on the processes of sperm maturation, indicating an adverse effect on epididymis (Perreault, 1998; Perreault & Cancel, 2001). The experimental group whose fathers were exposed to 10 mg of statin exhibited lower sperm motility, diminished sperm production and reduced relative sperm number in the epididymis caput/corpus and cauda. The other experimental groups showed sperm counts and motility similar to the controls. The rate of sperm head and tail abnormalities did not exhibit differences among the groups.

Under insufficient androgen stimulation epididymis epithelium regresses, send death signals to the lumen changing the composition of the luminal fluid and activates ubiquitin-proteasome system and DNAses, leading to sperm degradation and sperm disappearing (Jones, 2004). Indeed, reduced androgen levels were accompanied by diminished sperm number in the epididymis from the group whose fathers were treated with the higher dose of statin.

The amount of sperm DNA damage is another parameter assessed in toxicological studies on male reproduction (Perreault and Cancel, 2001). Increased sperm DNA damage is related to alterations on embryonic fate and early embryo development (Hales et al., 2005; Marchetti et al., 2003). Previous study has reported that androgen depletion is directly associated with augmented DNA fragmentation (Stanton et al., 2012), and in addition, statin exposure has been also associated with DNA damage (Coetsee et al., 2008; Leite et al., 2017a). Male offspring of rosuvastatin-exposed groups showed decreased testosterone concentrations and increased DNA damage in a dose-dependent manner. These results suggest that rosuvastatin can induce an indirect effect on genome stability, which is transmitted through the germ cell line of exposed parents (Leite et al., 2017a) to their offspring, i.e. epigenetics events. The epigenome is comprised of the modifications made in gene expression by changing DNA and histone structure without changing the DNA sequence itself (Rajender et al., 2011). Epigenetic processes include actions such as DNA methylation, post-translational histone modifications and chromatin remodeling (Rajender et al., 2011). These changes can have short- or long-term effects and might be transgenerational (Rajender et al., 2011).

Several studies reported sperm epigenetic changes compromising embryo development or causing diseases later in life, such as reproductive disorders (Favareto et al., 2011; Ly et al., 2017; Schagdarsurengin and Steger, 2016; Silva et al., 2016; Stuppia et al., 2015). Previous study has associated increased DNA damage caused by oxidative stress with aberrant global methylation (Rajender et al., 2011).

In this context, a previous study has proposed a mechanism wherein a radiation-exposure signal could be inherited through sperm in an epigenetic manner, perhaps by changes in DNA methylation (Dubrova et al., 2000). This epigenetic signal could influence expression patterns of genes involved in DNA repair or cell cycle checkpoints in the offspring of irradiated males, such changes could subsequently lead to an accumulation of unrepaired DNA damage and an increase in levels of mutations (Dubrova et al., 2000).

The literature has been suggested that statins can induce epigenetic changes; however, there are scarce available data about rosuvastatin (Allen and Mamotte, 2017). Thus, considering the transgenerational DNA damage observed in the offspring from rosuvastatin-exposed groups, we speculated that this lesion triggers an epigenetic signal that influences DNA repair and can survive the reprogramming of DNA methylation during spermatogenesis and early development (Heard and Martienssen, 2014), then subsequent activation of this signal in the germ line could lead to the observed transgenerational mutagenesis, such as was suggested by Dubrova et al (2000) on radiation studies. In this case, considering germline reprogramming fails, epigenetic marks can be retained, and could be transmitted from one generation to the next (Heard and Martienssen, 2014).

Interestingly, albeit vitamin C was not able to protect F0 from rosuvastin-induced DNA damage by alkaline comet assay (Leite et al 2017a), its F1 shows low levels of DNA lesions, similar that those from negative control (present study). Considering an epigenetics pathway, the vitamin C could erase epigenetic marks on DNA and enhance reprogramming (Hore et al., 2016). In this way, the methylation induced by rosuvastatin could be demethylated by vitamin C supplementation. In fact, ascorbic acid, named ascorbate in its intracellular form, regulates DNA demethylation, since it is an essential cofactor for Ten-eleven translocation (TET) dioxygenases and regulates histone demethylation because it is an important cofactor for Jumonji C-domain-containing histone demethylases (Young et al., 2015).

Testosterone is an essential steroid hormone for males because increases nitric oxide production, modifies neuronal excitability and neurotransmitters releasing in different brain regions, thus acting on central nervous system as a facilitator for the beginning of male sexual behavior (Gerardin et al., 2005; Hull et al., 1999). In this study, the parameters evaluated on sexual behavior testing showed to be similar among the groups; despite the male offspring from rosuvastatin-treated rats presents lower testosterone concentrations, which were not sufficient to affect this parameter. Moreover, reproductive performance and fertility test of the male offspring did not show differences among the groups for the assessed parameters.

Final body weight evaluation provides important information about the health condition of the animals (Clegg et al., 2001). In addition to this, reproductive organ weights supplies information about the reproductive system (Clegg et al., 2001) and vital organ weights may provide a general health condition of these organs. In this work, reproductive and vital organ weights, as well as final body weight showed no differences among the experimental groups.

This is the first study reporting reproductive damage in the male offspring in consequence of paternal exposure to rosuvastatin, besides to report a protecting role of paternal supplementation with ascorbic acid on ameliorating the reproductive impairment on male offspring. The findings in this work suggest that the reproductive adverse effects in the male offspring are related to augmented paternal sperm DNA fragmentation, as previously described (Leite et al., 2017a), besides a possible sperm epigenetic change on parental generation. Additional studies are necessary to clarify the molecular mechanisms involved on reproductive impairment in the male offspring mediated by paternal sperm epigenetics changes.

## 5. Conclusions

In conclusion, paternal exposure to rosuvastatin induced an impairment in the morphology and physiology of the reproductive system in the male offspring, represented by the increased frequency of germ cells death in the testis, augmented sperm DNA damage, hyperplasia of clear cells on cauda epididymis, reduced sperm counts, lower sperm motility and hormonal changes. The decreased testosterone levels in the plasma and testis, associated with the higher levels of LH suggest that paternal exposure to statin affected the pituitary-gonadal axis. Paternal supplementation with ascorbic acid played a preventive role in the male offspring against reproductive impairment provoked by paternal exposure to rosuvastatin. Ascorbic acid, when administered to the fathers, was able to protect sperm somehow, thus alleviating the reproductive impairment in their male offspring.

**Declaration of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgments**

The authors are grateful to São Paulo Research Foundation (FAPESP) (Grants #013/22495-1 and #014/13659-3), National Council for Scientific and Technological Development (CNPq) (Grant 308842/2013-8) and Coordination for the Improvement of Higher Education Personnel (CAPES) for their financial support, to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for his excellent technical assistance, to Dr. Daisy Maria Fávero Salvadori, from the Department of Pathology, Medical School, UNESP, Botucatu/SP – Brazil, for her relevant contributions with the sperm comet assay and to Dr. Ruither de Oliveira Gomes Carolino of the Department of Morphology, Physiology and Basic Pathology of the School of Dentristy of Ribeirão Preto, USP, for the hormones measurements.

**References**

Adamopoulos, D.A., Vassilopoulos, P., Kontogeorgos, L., Kapolla, N., 1990. Germinal epithelium changes in sexually immature rabbits treated with intratesticular testosterone implants. Andrologia 22, 557–565.

Agarwal, A., Prabhakaran, S.A., Said, T.M., 2005. Prevention of Oxidative Stress Injury to Sperm. J. Androl. 26, 654–660. doi:10.2164/jandrol.05016

Ahlenius, S., Larsson, K., 1984. Apomorphine and haloperidol-induced effects on male-rat sexual-behavior - No evidence for actions due to stimulation of central dopamine autoreceptors. Pharmacol. Biochem. Behav. 21, 463–466.

Allen, S.C., Mamotte, C.D.S., 2017. Pleiotropic and Adverse Effects of Statins—Do Epigenetics

Play a Role? J. Pharmacol. Exp. Ther. 362, 319–326. doi:10.1124/jpet.117.242081

Clegg, E.D., Perreault, S.D., Klinefelter, G.R., 2001. Assessment of male reproductive toxicology, in: Hayes, A.. (Ed.), Principles and Methods of Toxicology. Taylor & Francis, Philadelphia., pp. 1263–1299.

Coetsee, T.N., Pretorius, P.J., Terre'Blanche, G., Bergh, J.J., 2008. Investigating the potential neuroprotective effects of statins on DNA damage in mouse striatum. Food Chem. Toxicol. 46, 3186–3192. doi:10.1016/j.fct.2008.07.006

Cook, S., Kavey, R.E.W., 2011. Dyslipidemia and pediatric obesity. Pediatr. Clin. North Am. 58, 1363–1373. doi:10.1016/j.pcl.2011.09.003

Corsetti, G., Stacchiotti, A., Tedesco, L., D'Antona, G., Pasini, E., Dioguardi, F.S., Nisoli, E., Rezzani, R., 2011. Essential amino acid supplementation decreases liver damage induced by chronic ethanol consumption in rats. Int. J. Immunopathol. Pharmacol. 24, 611–619.

De Gendt, K., Swinnen, J. V, Saunders, P.T.K., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M., Verhoeven, G., 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. U. S. A. 101, 1327–32. doi:10.1073/pnas.0308114100

Dubrova, Y.E., Plumb, M., Gutierrez, B., Boulton, E., Jeffreys, A.J., 2000. Genome stability: Transgenerational mutation by radiation. Nature 405, 37–37. doi:10.1038/35011135

Eskenazi, B., Kidd, S.A., Marks, A.R., Sloter, E., Block, G., Wyrobek, A.J., 2005. Antioxidant intake is associated with semen quality in healthy men. Hum. Reprod. 20, 1006–1012. doi:10.1093/humrep/deh725

Favareto, A.P.A., de Toledo, F.C., Kempinas, W.D.G., 2011. Paternal treatment with cisplatin impairs reproduction of adult male offspring in rats. Reprod. Toxicol. 32, 425–433. doi:10.1016/j.reprotox.2011.10.003

Fernandes, G.S.A., Fernandez, C.D.B., Campos, K.E., Damasceno, D.C., Anselmo-Franci, J.A., Kempinas, W.D.G., 2011a. Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. Reprod. Biol. Endocrinol. 100, 1–9. doi:10.1186/1477-7827-9-100

Fernandes, G.S.A., Gerardin, D.C.C., Assumpção, T.A., Campos, K.E., Damasceno, D.C., Pereira, O.C.M., Kempinas, W.D.G., 2011b. Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats? Pharmacol. Reports 63,

983–991.

Filler, R., 1993. Methods for evaluation of rats epididymal sperm morphology, in: Chapin, R.E., Heindel, J.H. (Eds.), Male Reproductive Toxicology. Press, Academic, San Diego, pp. 334–343.

Gerardin, D.C.C., Pereira, O.C.M., Kempinas, W.G., Florio, J.C., Moreira, E.G., Bernardi, M.M., 2005. Sexual behavior, neuroendocrine, and neurochemical aspects in male rats exposed prenatally to stress. Physiol. Behav. 84, 97–104. doi:10.1016/j.physbeh.2004.10.014

Hales, B.F., Aguilar-Mahecha, A., Robaire, B., 2005. The stress response in gametes and embryos after paternal chemical exposures. Toxicol. Appl. Pharmacol. 207, 514–520. doi:10.1016/j.taap.2004.12.021

Heard, E., Martienssen, R.A., 2014. Transgenerational epigenetic inheritance: Myths and mechanisms. Cell 157, 95–109. doi:10.1016/j.cell.2014.02.045

Hermo, L., Barin, K., Robaire, B., 1992. Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. Anat. Rec. 233, 205–228. doi:10.1002/ar.1092330205

Holdgate, G.A., Ward, W.H.J., McTaggart, F., 2003. Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. Biochem. Soc. Trans. 31, 528–531. doi:10.1042/BST0310528

Hore, T.A., von Meyenn, F., Ravichandran, M., Bachman, M., Ficz, G., Oxley, D., Santos, F., Balasubramanian, S., Jurkowski, T.P., Reik, W., 2016. Retinol and ascorbate drive erasure of epigenetic memory and enhance reprogramming to naïve pluripotency by complementary mechanisms. Proc. Natl. Acad. Sci. 113, 12202–12207. doi:10.1073/pnas.1608679113

Hull, E.M., Lorrain, D.S., Du, J., Matuszewich, L., Lumley, L.A., Putnam, S.K., Moses, J., 1999. Hormone-neurotransmitter interactions in the control of sexual behavior. Behav. Brain Res. 105, 105–116. doi:10.1016/S0166-4328(99)00086-8

Istvan, E.S., Deisenhofer, J., 2001. Structural mechanism for statin inhibition of HMG-CoA reductase. Science (80-. ). 292, 1160–1164. doi:10.1126/science.1059344

Izar, M.C.D.O., Fonseca, M.I.H., Fonseca, F.A.H., 2011. Dyslipidemias. Rev. Bras. Med. 68, 59–73.

Jiménez, E.G., Ferre, J.A., 2011. Estatinas : Características y Efectos sobre el Control Lipídico en el Niño y Adolescente Obeso. Rev. Clínica Med. Fam. 4, 69–75.

Jones, R., 2004. Sperm survival versus degradation in the mammalian epididymis : A hypothesis. Biol. Reprod. 71, 1405–1411. doi:10.1095/biolreprod.104.031252

Kempinas, W.D.G., Klinefelter, G.R., 2015. Interpreting histopathology in the epididymis. Spermatogenesis 4, e979114. doi:10.4161/21565562.2014.979114

Kwiterovich, P.O., 2008a. Recognition and management of dyslipidemia in children and adolescents. J. Clin. Endocrinol. Metab. 93, 4200–4209. doi:10.1210/jc.2008-1270

Kwiterovich, P.O., 2008b. Clinical and laboratory assessment of cardiovascular risk in children: Guidelines for screening, evaluation, and treatment. J. Clin. Lipidol. 2, 248–266. doi:10.1016/j.jacl.2008.06.003

Leblond, C.P., Clermont, Y., 1952. Spermiogenesis of rat, mouse,hamster and guinea pig as revealed by the “periodic acid-fuchsin sulfurous acid” technique. Am. J. Anat. 90, 167–215.

Leite, G.A.A., Figueiredo, T.M., Pacheco, T.L., Sanabria, M., Silva, P.V., Fernandes, F.H., Kempinas, W.D.G., 2017a. Vitamin C partially prevents reproductive damage in adult male rats exposed to rosuvastatin during prepuberty. Food Chem. Toxicol.

Leite, G.A.A., Figueiredo, T.M., Sanabria, M., Dias, A.F.M.G., Silva, P.V., Martins Junior, A. da C., Barbosa Junior, F., Kempinas, W.D.G., 2017b. Ascorbic acid supplementation partially prevents the delayed reproductive development in juvenile male rats exposed to rosuvastatin since prepuberty. Reprod. Toxicol. doi:10.1016/j.reprotox.2017.07.006

Leite, G.A.A., Rosa, J. de L., Sanabria, M., Cavariani, M.M., Franci, J.A.A., Pinheiro, P.F.F., Kempinas, W.D.G., 2014. Delayed reproductive development in pubertal male rats exposed to the hypolipemiant agent rosuvastatin since prepuberty. Reprod. Toxicol. 44, 93–103. doi:10.1016/j.reprotox.2014.01.004

Ludman, A., Venugopal, V., Yellon, D.M., Hausenloy, D.J., 2009. Statins and cardioprotection - More than just lipid lowering? Pharmacol. Ther. 122, 30–43. doi:10.1016/j.pharmthera.2009.01.002

Ly, L., Chan, D., Aarabi, M., Landry, M., Behan, N.A., Macfarlane, A.J., Trasler, J., 2017. Intergenerational impact of paternal lifetime exposures to both folic acid de fi ciency and supplementation on reproductive outcomes and imprinted gene methylation. Mol. Hum. Reprod. 1, 1–17. doi:10.1093/molehr/gax029

Mantovani, A., Fucic, A., 2014. Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. Reprod. Toxicol. 44, 15–22. doi:10.1016/j.reprotox.2013.06.002

Marchetti, F., Bishop, J.B., Consentino, L., Moore II, L., Wyrobek, A.J., 2003. Paternally Transmitted Chromosomal Aberrations in Mouse Zygotes Determine Their Embryonic Fate. Biol. Reprod. 70, 616–624. doi:10.1095/biolreprod.103.023044

Marty, M.S., Chapin, R.E., Parks, L.G., Thorsrud, B.A., 2003. Development and Maturation of the Male Reproductive System. Birth Defects Res. Part B - Dev. Reprod. Toxicol. 68, 125–136. doi:10.1002/bdrb.10015

McTaggart, F., 2003. Comparative pharmacology of rosuvastatin. Atheroscler. Suppl. 4, 9–14. doi:10.1016/S1567-5688(03)00004-7

Mukhopadhyay, P.K., Dey, A., Mukherjee, S., Pradhan, N.K., 2013. The effect of coadministration of alpha-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. J. Basic Clin. Physiol. Pharmacol. 24, 245–253. doi:10.1515/jbcpp-2012-0039

Pandir, D., Kara, O., Kara, M., 2014. Protective effect of bilberry (Vaccinium myrtillus L.) on cisplatin induced ovarian damage in rat. Cytotechnology 66, 677–685. doi:10.1007/s10616-013-9621-z

Perobelli, J.E., Alves, T.R., de Toledo, F.C., Fernandez, C.D.B., Anselmo-Franci, J.A., Klinefelter, G.R., Kempinas, W.D.G., 2012. Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. Reprod. Toxicol. 33, 308–315. doi:10.1016/j.reprotox.2011.12.011

Perobelli, J.E., Patrão, M.T.C.C., Fernandez, C.D.B., Sanabria, M., Klinefelter, G.R., Avellar, M.C.W., Kempinas, W.D.G., 2013. Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. Reprod. Toxicol. 38, 65–71. doi:10.1016/j.reprotox.2013.03.004

Perreault, S.D., 1998. Gamete toxicology: the impact of new technologies, in: Dekker, M. (Ed.), Reproductive and Developmental Toxicology. KS. Korach, New York, pp. 635–654.

Perreault, S.D., Cancel, A.M., 2001. Significance of incorporating measures of sperm production and function into rat toxicology studies. Reproduction 121, 207–216. doi:10.1530/reprod/121.2.207

Pihlajamaa, P., Sahu, B., Jänne, O.A., 2015. Determinants of receptor- and tissue-specific actions in androgen signaling. Endocr. Rev. 36, 357–384. doi:10.1210/er.2015-1034

Rajender, S., Avery, K., Agarwal, A., 2011. Epigenetics , spermatogenesis and male infertility.

Mutat. Res. / Rev. Mutat. Res. 727, 62–71. doi:10.1016/j.mrrev.2011.04.002

Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J. 22, 659–661. doi:10.1096/fj.07-9574LSF

Robb, G.W., Amann, R.P., Killian, G.J., 1978. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J. Reprod. Fertil. 54, 103–107. doi:10.1530/jrf.0.0540103

Ross, J.L., 2016. Statins in the Management of Pediatric Dyslipidemia. J. Pediatr. Nurs. 31, 723–735. doi:10.1016/j.pedn.2016.07.004

Schagdarsurengin, U., Steger, K., 2016. Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. Nat. Rev. Urolology 13, 584–595. doi:10.1038/nrurol.2016.157

Seed, J., Chapin, R.E., Clegg, E.D., Dostal, L.A., Foote, R.H., Hurtt, M.E., Klinefelter, G.R., Makris, S.L., Perreault, S.D., Schrader, S., Seyler, D., Sprando, R., Treinen, K.A., Veeramachaneni, D.N.R., Wise, L.D., 1996. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: A consensus report. Reprod. Toxicol. 10, 237–244. doi:10.1016/0890-6238(96)00028-7

Shrilatha, B., Muralidhara, 2007. Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. Reprod. Toxicol. 23, 578–587. doi:10.1016/j.reprotox.2007.02.001

Silva, P.V., da Silva, R.F., Borges, C. dos S., Cavariani, M.M., de Almeida Francia, C.C.D., Junior, F.B., De Grava Kempinas, W., 2016. Sexual differentiation and reproductive development of female rat offspring after paternal exposure to the anti-tumor pharmaceutical cisplatin. Reprod. Toxicol. 60, 112–122. doi:10.1016/j.reprotox.2016.02.005

Sönmez, M., Turk, G., Yuce, A., 2005. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology 63, 2063–2072. doi:10.1016/j.theriogenology.2004.10.003

Sooriyaarachchi, M., Narendran, A., Gailer, J., 2012. The effect of sodium thiosulfate on the metabolism of cis-platin in human plasma in vitro. Metallomics 4, 960–967. doi:10.1039/c2mt20076g

Stanton, P.G., Sluka, P., Foo, C.F.H., Stephens, A.N., Smith, A.I., Mclachlan, R.I., Donnell, L.O., 2012. Proteomic changes in rat spermatogenesis in response to in vivo androgen manipulation: Impact on meiotic cells. PLoS One 7, 1–19.

doi:10.1371/journal.pone.0041718

Stoker, T.E., Laws, S.C., Guidici, D.L., Cooper, R.L., 2000. The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function. Toxicol. Sci. 58, 50–59. doi:10.1093/toxsci/58.1.50

Stuppia, L., Franzago, M., Ballerini, P., Gatta, V., Antonucci, I., 2015. Epigenetics and male reproduction : the consequences of paternal lifestyle on fertility , embryo development , and children lifetime health. Clin. Epigenetics 7, 1–15. doi:10.1186/s13148-015-0155-4

Tandon, V., Bano, G., Khajuria, V., Parihar, A., Gupta, S., 2005. Pleiotropic effects of statins. Indian J. Pharmacol. 37, 77–85. doi:10.5772/23502

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel / comet assay : Guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221.

Vaughan, C.J., Gotto Jr, A.M., 2004. Update on Statins: 2003. Circulation 110, 886–892. doi:10.1161/01.CIR.0000139312.10076.BA

Wiegman, A., Gidding, S.S., Watts, G.F., Chapman, M.J., Ginsberg, H.N., Cuchel, M., Ose, L., Averna, M., Boileau, C., Borén, J., Bruckert, E., Catapano, A.L., Defesche, J.C., Descamps, O.S., Hegele, R.A., Hovingh, G.K., Humphries, S.E., Kovanen, P.T., Kuivenhoven, J.A., Masana, L., Nordestgaard, B.G., Pajukanta, P., Parhofer, K.G., Raal, F.J., Ray, K.K., Santos, R.D., Stalenhoef, A.F.H., Steinhagen- Thiessen, E., Stroes, E.S., Taskinen, M.R., Tybjealigrg-Hansen, A., Wiklund, O., Catapano, A.L., Defesche, J.C., Descamps, O.S., Gidding, S.S., Ginsberg, H.N., Hegele, R.A., Humphries, S.E., Kovanen, P.T., Nordestgaard, B.G., Parhofer, K.G., Raal, F.J., Ray, K.K., Santos, R.D., Stalenhoef, A.F.H., Steinhagen-Thiessen, E., Stroes, E.S., Watts, G.F., 2015. Familial hypercholesterolaemia in children and adolescents: Gaining decades of life by optimizing detection and treatment. Eur. Heart J. 36, 2425–2437. doi:10.1093/eurheartj/ehv157

Zhao, X., Cheng, Z., Zhu, Y., Li, S., Zhang, L., Luo, Y., 2015. Effects of paternal cadmium exposure on the sperm quality of male rats and the neurobehavioral system of their offspring. Exp. Ther. Med. 10, 2356–2360. doi:10.3892/etm.2015.2777

**Figure captions**

**Figure 1.** Photomicrography of testicular sections in the male offspring from experimental groups on postnatal day (PND) 110. Observe the presence of acidophilic germ cells (arrows) in the seminiferous epithelium of the male offspring from rosuvastatin-exposed groups. Hematoxylin and Eosin (HE). Scale bar = 100µm or 50µm.

**Figure 2.** Photomicrography of TUNEL staining in testicular sections in the male offspring from experimental groups on postnatal day (PND) 110. Observe the presence of TUNEL-marked germ cells (arrows) in the seminiferous epithelium of the male offspring from rosuvastatin-exposed groups. Scale bar = 50µm.

**Figure 3.** Photomicrography of epididymal sections in the male offspring from the experimental groups on PND 110. Observe an apparent hyperplasia of clear cells in the epididymal proximal cauda of the male offspring from rosuvastatin-exposed groups (arrows). Hematoxylin and Eosin (HE). Scale bar = 100µm.

**Figure 4.** Sperm motility in rat male offspring from the experimental groups at 110 days of age. Values expressed as median and interquartile intervals. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 5.** Sperm DNA fragmentation (Comet assay) in rat male offspring from the experimental groups, observed by percentage of tail intensity. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 6.** Hormonal concentrations (ng/ml) in rat male offspring at 110 days of age. Values expressed as mean ± standard error of mean (SEM). Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Table 1.** Testicular histopathological evaluation and spermatogenesis kinetics in the male offspring from experimental groups on post-natal day (PND) 110.

| | Experimental Groups (n=9 or 10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Normal seminiferous tubules (%) | 97.00 (96.00 – 98.50) $^{a}$ | 95.25 (94.50 – 97.00) $^{b}$ | 94.50 (94.00 – 95.50) $^{b}$ | 97.50 (97.00 – 98.50) $^{a}$ | 96.25 (95.50 – 98.50) $^{a}$ | 96.50 (96.00 – 98.00) $^{a}$ |
| Degeneration of germ cells (% of tubules) | 1.75 (0.50 – 3.00) $^{a}$ | 4.00 (2.50 – 5.50) $^{b}$ | 4.50 (3.50 – 5.00) $^{b}$ | 1.75 (1.50 – 3.00) $^{a}$ | 1.50 (3.00 – 4.50) $^{a}$ | 2.50 (1.50 – 3.50) $^{a}$ |
| Stages I – VI (%) | 34.00 (30.00 – 42.00) $^{a}$ | 36.00 (33.00 – 41.00) $^{a}$ | 33.00 (29.00 – 39.00) $^{a}$ | 34.50 (32.00 – 43.00) $^{a}$ | 35.50 (28.00 – 37.00) $^{a}$ | 35.00 (30.00 –36.00) $^{a}$ |
| Stages VII – VIII (%) | 31.00 (26.00 – 36.00) $^{a}$ | 29.00 (26.00 – 31.00) $^{a}$ | 32.50 (25.00 – 35.00) $^{a}$ | 30.50 (25.00 – 34.00) $^{a}$ | 29.00 (26.00 –35.00) $^{a}$ | 32.00 (28.00 – 38.00) $^{a}$ |
| Stages IX – XIII (%) | 29.50 (25.00 – 36.00) $^{a}$ | 29.50 (21.00 – 35.00) $^{a}$ | 30.50 (25.00 – 33.00) $^{a}$ | 29.00 (27.00 – 32.00) $^{a}$ | 29.50 (24.00 – 38.00) $^{a}$ | 30.00 (23.00 – 35.00) $^{a}$ |
| Stage XIV (%) | 5.00 (2.00 – 8.00) $^{a}$ | 6.00 (2.00 – 7.00) $^{a}$ | 6.00 (3.00 – 7.00) $^{a}$ | 5.00 (3.00 – 8.00) $^{a}$ | 6.00 (3.00 – 7.00) $^{a}$ | 5.00 (2.00 – 8.00) $^{a}$ |

Values expressed as median and interquartile intervals, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 2.** Rate of seminiferous tubules with TUNEL-positive germ cells in relation to the total of seminiferous tubules in the same categories of stages of seminiferous epithelium in the male offspring from experimental groups on PND 110.

| | Experimental Groups (n= 5) | | | | | |
|---|---|---|---|---|---|---|
| **Parameters (%)** | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Total of seminiferous tubules with TUNEL-positive germ cells | 3.00 (2.00 – 4.00) $^{a}$ | 13.00 (8.00 – 13.50) $^{b}$ | 9.00 (7.50 – 11.50) $^{b}$ | 4.00 (2.50 – 4.50) $^{a}$ | 5.00 (3.00 – 5.00) $^{a}$ | 3.00 (2.50 – 4.50) $^{a}$ |
| Stages I-VI | 5.13 (2.57 – 6.12) $^{a}$ | 15.00 (12.87 – 20.77) $^{b}$ | 11.63 (5.99 – 13.81) $^{c}$ | 4.76 (3.39 – 7.54) $^{a}$ | 6.12 (3.41 – 9.10) $^{a}$ | 6.25 (3.75 – 6.53) $^{a}$ |
| Stages VII-VIII | 0 (0 – 0) $^{a}$ | 0 (0 – 1.52) $^{a}$ | 0 (0 – 0) $^{a}$ | 0 (0 – 0) $^{a}$ | 0 (0 – 0) $^{a}$ | 0 (0 – 0) $^{a}$ |
| Stage IX-XIV | 3.70 (3.03 – 5.74) $^{a}$ | 15.38 (8.04 – 24.71) $^{b}$ | 20.00 (20.00 – 21.29) $^{b}$ | 4.76 (1.79 – 8.17) $^{a}$ | 4.00 (1.67 – 10.42) $^{a}$ | 3.70 (1.79 – 5.93) $^{a}$ |

Values expressed as median and interquartile intervals, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 3.** Sperm counts in the testis and epididymis in the male offspring from experimental groups on PND 110.

| Sperm counts | Experimental Groups (n = 9) | | | | | |
|---|---|---|---|---|---|---|
| | Control | 3 mg | 10 mg | AA | 3 + AA | 10 + AA |
| *Sperm counts in the testis* | | | | | | |
| Mature spermatid number ($10^6$/testis) | $195.30 \pm 2.55^{a}$ | $187.00 \pm 4.73^{a}$ | $172.70 \pm 3.30^{b}$ | $191.30 \pm 4.72^{a}$ | $193.30 \pm 3.31^{a}$ | $188.30 \pm 2.41^{a}$ |
| Mature spermatid number ($10^6$/g testis) | $118.10 \pm 2.67^{a}$ | $110.30 \pm 3.09^{ab}$ | $100.60 \pm 2.89^{b}$ | $114.50 \pm 3.46^{a}$ | $112.10 \pm 7.74^{a}$ | $111.60 \pm 3.62^{a}$ |
| Daily sperm production ($10^6$/testis/day) | $32.00 \pm 0.42^{a}$ | $30.66 \pm 0.74^{a}$ | $28.31 \pm 0.54^{b}$ | $31.36 \pm 0.77^{a}$ | $31.69 \pm 0.54^{a}$ | $30.87 \pm 0.40^{a}$ |
| Relative sperm production ($10^6$/g testis/day) | $19.36 \pm 0.44^{a}$ | $18.08 \pm 0.51^{ab}$ | $16.49 \pm 0.47^{b}$ | $18.77 \pm 0.57^{a}$ | $18.38 \pm 0.40^{a}$ | $18.30 \pm 0.59^{a}$ |
| ***Sperm counts in the epididymis*** | | | | | | |
| Sperm number in the caput/corpus ($x10^6$/organ) | $126.70 \pm 4.32^{a}$ | $127.70 \pm 6.80^{a}$ | $117.00 \pm 5.38^{a}$ | $127.80 \pm 5.31^{a}$ | $123.20 \pm 3.72^{a}$ | $124.80 \pm 3.66^{a}$ |
| Relative sperm number in the caput/corpus ($x10^6$/g/organ) | $448.10 \pm 18.38^{a}$ | $412.00 \pm 14.44^{a}$ | $379.20 \pm 15.28^{b}$ | $457.30 \pm 15.85^{a}$ | $439.40 \pm 18.12^{a}$ | $430.00 \pm 16.10^{a}$ |
| Sperm number in the cauda ($x10^6$/organ) | $245.90 \pm 4.12^{a}$ | $249.10 \pm 9.80^{a}$ | $228.54 \pm 5.67^{a}$ | $251.60 \pm 6.88^{a}$ | $255.00 \pm 8.07^{a}$ | $248.10 \pm 5.72^{a}$ |
| Relative sperm number in the cauda ($x10^6$/g/organ) | $1265.00 \pm 34.10^{a}$ | $1128.00 \pm 44.47^{a}$ | $1031.00 \pm 31.99^{b}$ | $1138.00 \pm 41.59^{a}$ | $1173.00 \pm 34.57^{a}$ | $1114.00 \pm 54.35^{a}$ |
| Sperm transit time in the caput/corpus (days) | $3.96 \pm 0.11^{a}$ | $4.15 \pm 0.14^{a}$ | $4.13 \pm 0.16^{a}$ | $4.08 \pm 0.12^{a}$ | $3.89 \pm 0.11^{a}$ | $4.05 \pm 0.12^{a}$ |
| Sperm transit time in the cauda (days) | $7.69 \pm 0.14^{a}$ | $8.11 \pm 0.19^{a}$ | $8.08 \pm 0.15^{a}$ | $7.82 \pm 0.22^{a}$ | $8.03 \pm 0.13^{a}$ | $8.04 \pm 0.16^{a}$ |
| Total sperm transit time (days) | $11.65 \pm 0.16^{a}$ | $12.27 \pm 0.30^{a}$ | $12.20 \pm 0.23^{a}$ | $11.89 \pm 0.27^{a}$ | $11.92 \pm 0.15^{a}$ | $12.09 \pm 0.23^{a}$ |

Values expressed as mean ± standard error of mean (SEM), $p < 0.05$. Two-way ANOVA followed by Bonferroni's test. Different letters indicate statistically significant differences among the groups.

**Table 4.** Sperm morphology in the male offspring from experimental groups on PND 110.

| Sperm morphology (%) | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Normal shaped sperm | 96.50 (96.00 – 98.25) | 95.00 (94.50 – 97.88) | 96.00 (94.25 – 96.75) | 95.75 (94.38 – 97.25) | 95.50 (94.38 – 97.25) | 95.25 (96.00 – 97.50) |
| Sperm head abnormalities | 2.00 (0.50 – 2.50) | 1.75 (1.13 – 2.50) | 1.50 (0.75 – 2.25) | 1.75 (0.88 – 3.13) | 2.00 (0.38 – 2.88) | 2.50 (1.00 – 3.50) |
| Sperm tail abnormalities | 1.50 (1.00 – 2.25) | 2.50 (1.00 – 4.25) | 2.50 (1.50 – 4.50) | 2.25 (0.50 – 3.13) | 2.00 (1.88 – 3.13) | 1.50 (1.00 - 2.25) |
| Sperm with cytoplasmic droplet | 14.00 (10.25 – 16.25) | 13.00 (12.13 – 14.38) | 13.50 (11.25 – 15.00) | 13.00 (12.00 – 17.00) | 13.00 (9.63 – 14.25) | 14.00 (9.50 – 16.00) |

Values expressed as median and interquartile intervals, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 5.** Sexual behavior test in the male offspring from experimental groups on PND 100.

| | Experimental Groups (n=8) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Number of mounts until de first intromission | 2.43 ± 0.61 | 4.33 ± 1.56 | 3.86 ± 1.10 | 3.25 ± 0.49 | 2.33 ± 0.50 | 2.25 ± 0.62 |
| Latency to the first intromission (s) | 345.78 ± 23.44 | 318.14 ± 29.58 | 382.43 ± 26.17 | 373.71 ± 29.40 | 349.78 ± 14.12 | 389.71 ± 23.78 |
| Number of intromissions until the first ejaculation | 19.22 ± 2.60 | 16.00 ± 2.56 | 17.71 ± 1.82 | 17.00 ± 3.28 | 18.80 ± 1.87 | 18.12 ± 3.00 |
| Latency to the first ejaculation (s) | 956.11 ± 91.73 | 845.00 ± 131.36 | 960.14 ± 135.08 | 990.75 ± 143.66 | 1112.22 ± 132.52 | 857.86 ± 90.87 |
| Latency to the first post-ejaculatory intromission (s) | 183.56 ± 50.30 | 213.62 ± 53.17 | 312.00 ± 87.08 | 189.78 ± 33.84 | 325.44 ± 57.40 | 291.62 ± 36.35 |
| Number of post-ejaculatory intromission | 7.78 ± 1.29 | 10.71 ± 1.11 | 9.57 ± 1.38 | 11.85 ± 2.63 | 7.78 ± 0.79 | 10.00 ± 1.85 |
| Number of ejaculations | 2.22 ± 0.22 | 2.25 ± 0.31 | 2.57 ± 0.30 | 2.12 ± 0.35 | 2.33 ± 0.33 | 2.57 ± 0.37 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 6.** Reproductive performance and fertility test in the male offspring from experimental groups on PND 110.

| | Experimental Groups (n=9 or 10) | | | | | |
|---|---|---|---|---|---|---|
| | Control | 3 mg | 10 mg | AA | 3 + AA | 10 + AA |
| Gestational rate (%) | 100% | 100% | 100% | 100% | 80% | 87.50% |
| [1]Fertility test (%) | 100 (100 – 100) | 100 (94.65 – 100) | 100 (100 – 100) | 100 (96.16 – 100) | 96.43(89.25 – 100) | 100 (91.67 – 100) |
| [1]Post-implantation loss (%) | 0 (0 – 8.33) | 9.09 (0 – 22.65) | 0 (0 – 8.33) | 0 (0 – 8.71) | 6.90 (0 – 12.79) | 0 (0 – 7.69) |
| [1]Sex ratio (M:F) | 1.00 (0.53 – 1.75) | 1.00 (1.00– 3.50) | 0.80 (0.67 – 1.20) | 0.67 (0.35 – 2.00) | 0.84 (0.58 – 1.25) | 0.72 (0.54 – 1.62) |
| [2]Body weight (GD20) | 342.24 ± 13.62 | 346.19 ± 11.06 | 324.36 ± 11.51 | 336.84 ± 12.38 | 365.62 ± 8.54 | 324.79 ± 14.88 |
| [2]Gravid uterus | 61.62 ± 3.56 | 53.58 ± 5.71 | 57.77 ± 2.22 | 59.84 ± 2.38 | 63.99 ± 2.40 | 56.97 ± 8.76 |
| [2]Male fetus weight | 3.42 ± 0.13 | 3.36 ± 0.11 | 3.46 ± 0.12 | 3.49 ± 0.08 | 3.38 ± 0.09 | 3.39 ± 0.12 |
| [2] Female fetus weight | 3.22 ± 0.12 | 3.17 ± 0.07 | 3.21 ± 0.11 | 3.28 ± 0.09 | 3.12 ± 0.07 | 3.09 ± 0.13 |
| [2]Placenta weight from male fetus | 0.56 ± 0.02 | 0.55 ± 0.04 | 0.55 ± 0.02 | 0.55 ± 0.01 | 0.55 ± 0.03 | 0.63 ± 0.04 |
| [2] Placenta weight from female fetus | 0.53 ± 0.02 | 0.52 ± 0.04 | 0.55 ± 0.02 | 0.56 ± 0.03 | 0.52 ± 0.03 | 0.60 ± 0.04 |

Values expressed as median and interquartile intervals[1] or mean ± SEM[2], $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 7.** Body weight and reproductive organ weights in the male offspring from experimental groups on PND 110.

| | **Experimental Groups (n=10)** | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Final body weight (g) | 452.30 ± 7.64 | 452.90 ± 10.29 | 429.60 ± 14.58 | 420.30 ± 8.09 | 439.80 ± 9.42 | 435.80 ± 10.93 |
| Testis (g) | 1.92 ± 0.05 | 2.13 ± 0.05 | 2.13 ± 0.07 | 2.04 ± 0.06 | 1.94 ± 0.04 | 1.99 ± 0.05 |
| Testis (g/100g BW) | 0.39 ± 0.01 | 0.40 ± 0.01 | 0.42 ± 0.01 | 0.42 ± 0.02 | 0.41 ± 0.01 | 0.41 ± 0.01 |
| Epididymis (mg) | 638.70 ± 15.66 | 661.80 ± 23.89 | 653.10 ± 15.16 | 637.10 ± 17.63 | 661.30 ± 16.78 | 642.60 ± 8.97 |
| Epididymis (mg/100g BW) | 141.40 ± 3.64 | 146.10 ± 4.05 | 152.80 ± 3.67 | 152.00 ± 4.80 | 150.40 ± 2.26 | 148.00 ± 3.46 |
| Vas deferens (mg) | 95.64 ± 3.96 | 90.16 ± 3.43 | 102.52 ± 4.12 | 95.95 ± 3.17 | 94.20 ± 2.66 | 91.93 ± 3.89 |
| Vas deferens (mg/100g BW) | 21.29 ± 1.16 | 19.97 ± 0.85 | 23.94 ± 0.84 | 22.86 ± 0.95 | 21.47 ± 0.66 | 21.17 ± 0.98 |
| Prostate (mg) | 549.70 ± 35.86 | 634.80 ± 47.44 | 587.80 ± 44.63 | 615.40 ± 32.58 | 609.50 ± 41.60 | 530.20 ± 36.64 |
| Prostate (mg/100g BW) | 122.70 ± 9.64 | 140.50 ± 10.74 | 136.10 ± 7.79 | 147.40 ± 9.47 | 137.90 ± 7.58 | 121.30 ± 6.48 |
| Full seminal gland (mg) | 1.48 ± 0.06 | 1.48 ± 0.06 | 1.55 ± 0.09 | 1.37 ± 0.08 | 1.43 ± 0.10 | 1.412 ± 0.10 |
| Full seminal gland (mg/100g BW) | 319.30 ± 19.90 | 328.20 ± 14.69 | 361.70 ± 17.06 | 328.20 ± 20.68 | 324.20 ± 17.30 | 324.80 ± 25.23 |
| Empty seminal gland (mg) | 527.40 ± 14.83 | 565.70 ± 30.55 | 550.70 ± 31.97 | 529.10 ± 39.41 | 549.70 ± 39.87 | 494.30 ± 15.84 |
| Empty seminal gland (mg/100g BW) | 117.00 ± 4.34 | 124.40 ± 4.59 | 128.70 ± 7.25 | 126.80 ± 10.52 | 124.60 ± 7.86 | 113.90 ± 4.37 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Table 8.** Vital organ weights in the male offspring from experimental groups on PND 110.

| | **Experimental Groups (n=10)** | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 + AA** | **10 + AA** |
| Pituitary (mg) | 10.80 ± 0.84 | 12.16 ± 0.68 | 10.46 ± 0.61 | 11.44 ± 0.50 | 11.23 ± 0.33 | 10.70 ± 0.61 |
| Pituitary (mg/100g BW) | 2.38 ± 0.18 | 2.69 ± 0.14 | 2.45 ± 0.14 | 2.72 ± 0.10 | 2.56 ± 0.08 | 2.48 ± 0.18 |
| Thyroid (mg) | 20.88 ± 1.23 | 21.71 ± 1.02 | 19.60 ± 0.79 | 19.35 ± 0.75 | 18.69 ± 1.45 | 19.36 ± 1.64 |
| Thyroid (mg/100g BW) | 4.62 ± 0.27 | 4.79 ± 0.17 | 4.60 ± 0.22 | 4.62 ± 0.19 | 4.24 ± 0.29 | 4.42 ± 0.32 |
| Liver (g) | 17.08 ± 0.52 | 16.96 ± 0.50 | 15.63 ± 0.79 | 15.41 ± 0.56 | 15.43 ± 0.70 | 15.72 ± 0.71 |
| Liver (g/100g BW) | 3.77 ± 0.07 | 3.75 ± 0.08 | 3.63 ± 0.12 | 3.66 ± 0.09 | 3.50 ± 0.12 | 3.61 ± 0.12 |
| Adrenal (mg) | 36.46 ± 2.25 | 38.24 ± 0.66 | 34.78 ± 2.32 | 33.53 ± 1.24 | 34.71 ± 1.80 | 33.94 ± 1.49 |
| Adrenal (mg/100g BW) | 8.03 ± 0.43 | 8.46 ± 0.17 | 8.11 ± 0.49 | 8.01 ± 0.33 | 7.89 ± 0.36 | 7.83 ± 0.41 |
| Kidney (g) | 1.69 ± 0.04 | 1.65 ± 0.03 | 1.60 ± 0.05 | 1.53 ± 0.04 | 1.59 ± 0.03 | 1.65 ± 0.03 |
| Kidney (g/100g BW) | 0.37 ± 0.01 | 0.36 ± 0.01 | 0.37 ± 0.01 | 0.36 ± 0.01 | 0.36 ± 0.01 | 0.38 ± 0.01 |
| Brain (g) | 2.03 ± 0.03 | 1.99 ± 0.04 | 2.01 ± 0.04 | 1.98 ± 0.03 | 2.01 ± 0.04 | 1.99 ± 0.02 |
| Brain (g/100g BW) | 0.45 ± 0.01 | 0.44 ± 0.01 | 0.47 ± 0.01 | 0.47 ± 0.01 | 0.46 ± 0.01 | 0.46 ± 0.01 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Figure 1.**

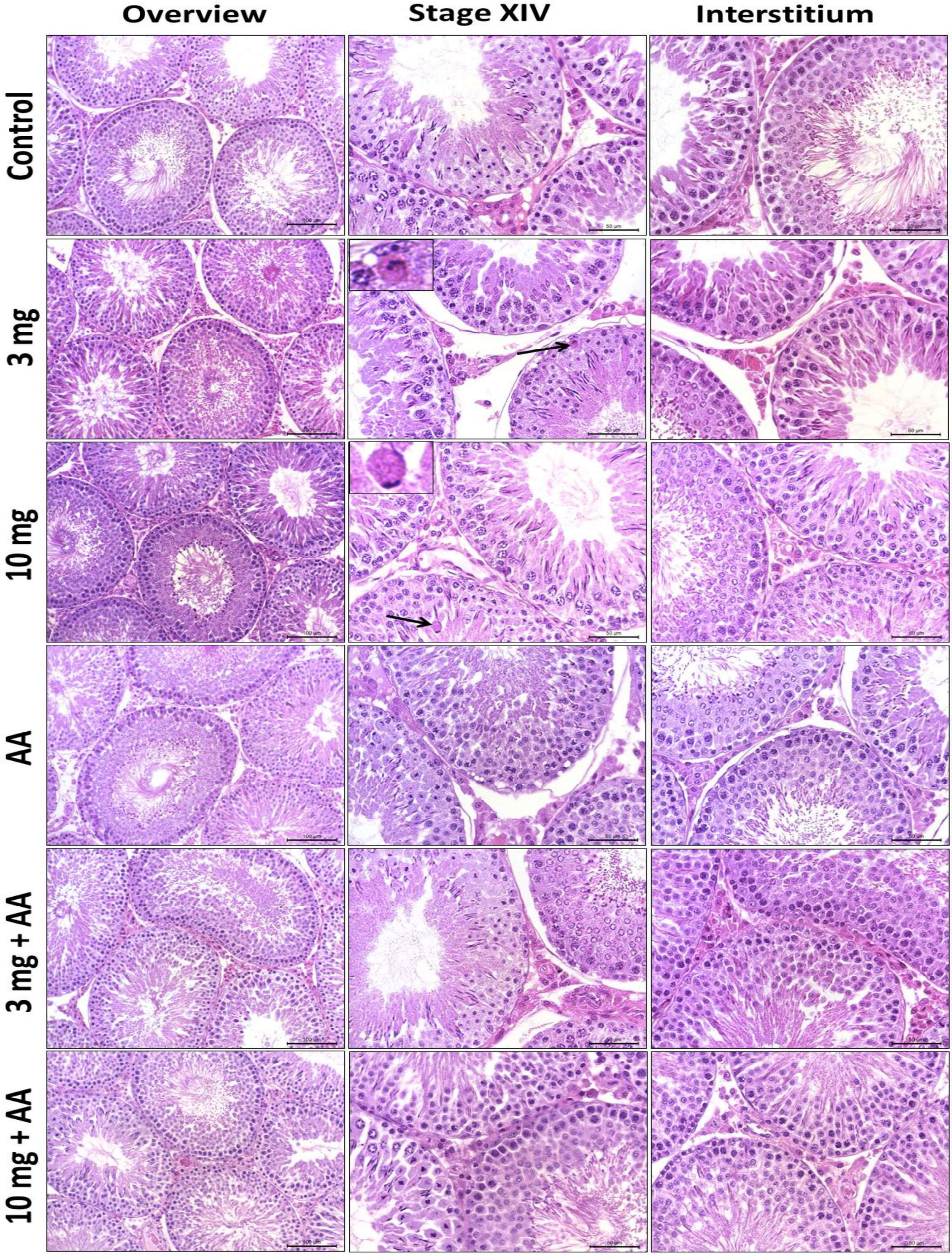

Overview
Stage XIV
Interstitium
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 2.**

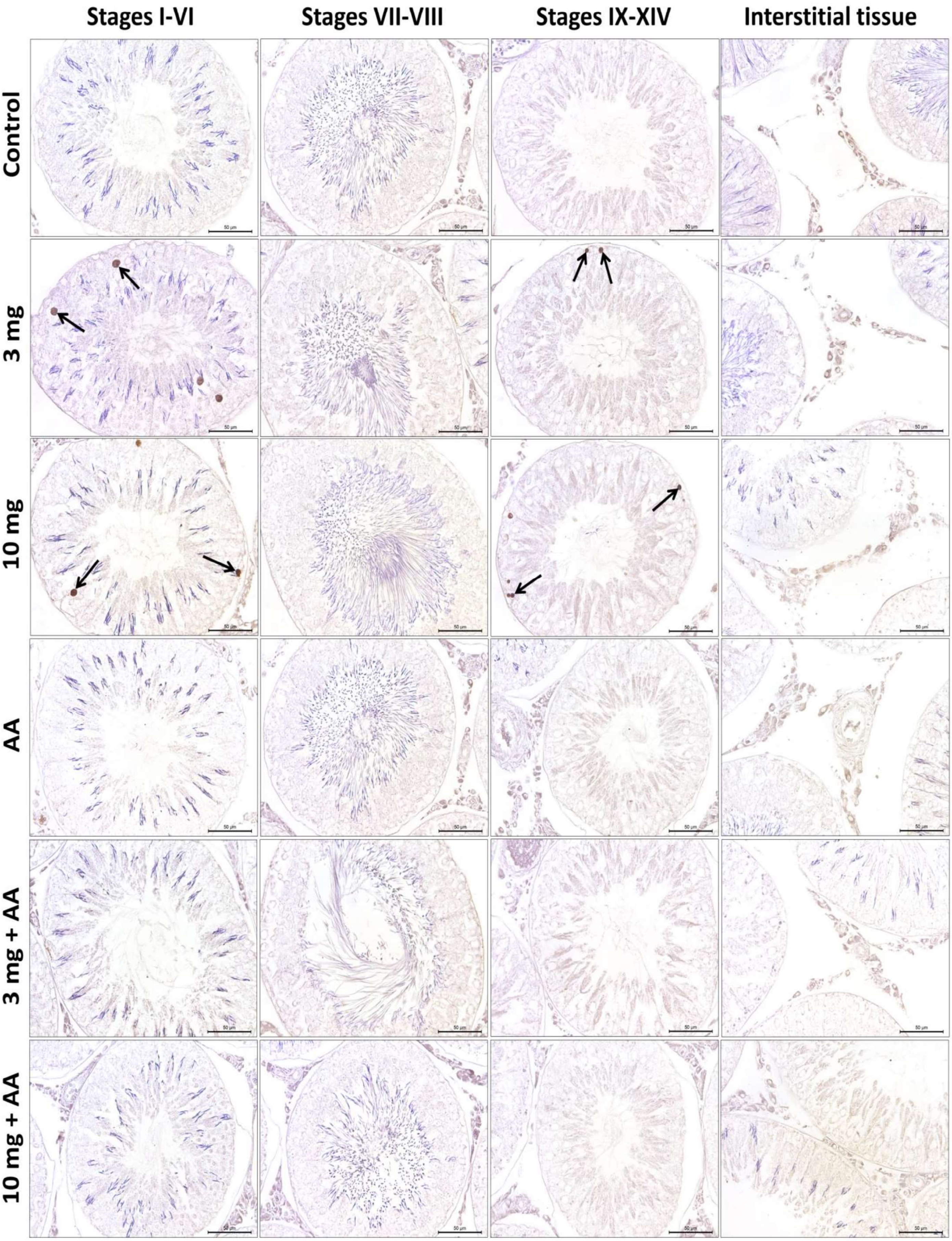

Stages I-VI
Stages VII-VIII
Stages IX-XIV
Interstitial tissue
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
50 µm

**Figure 3.**

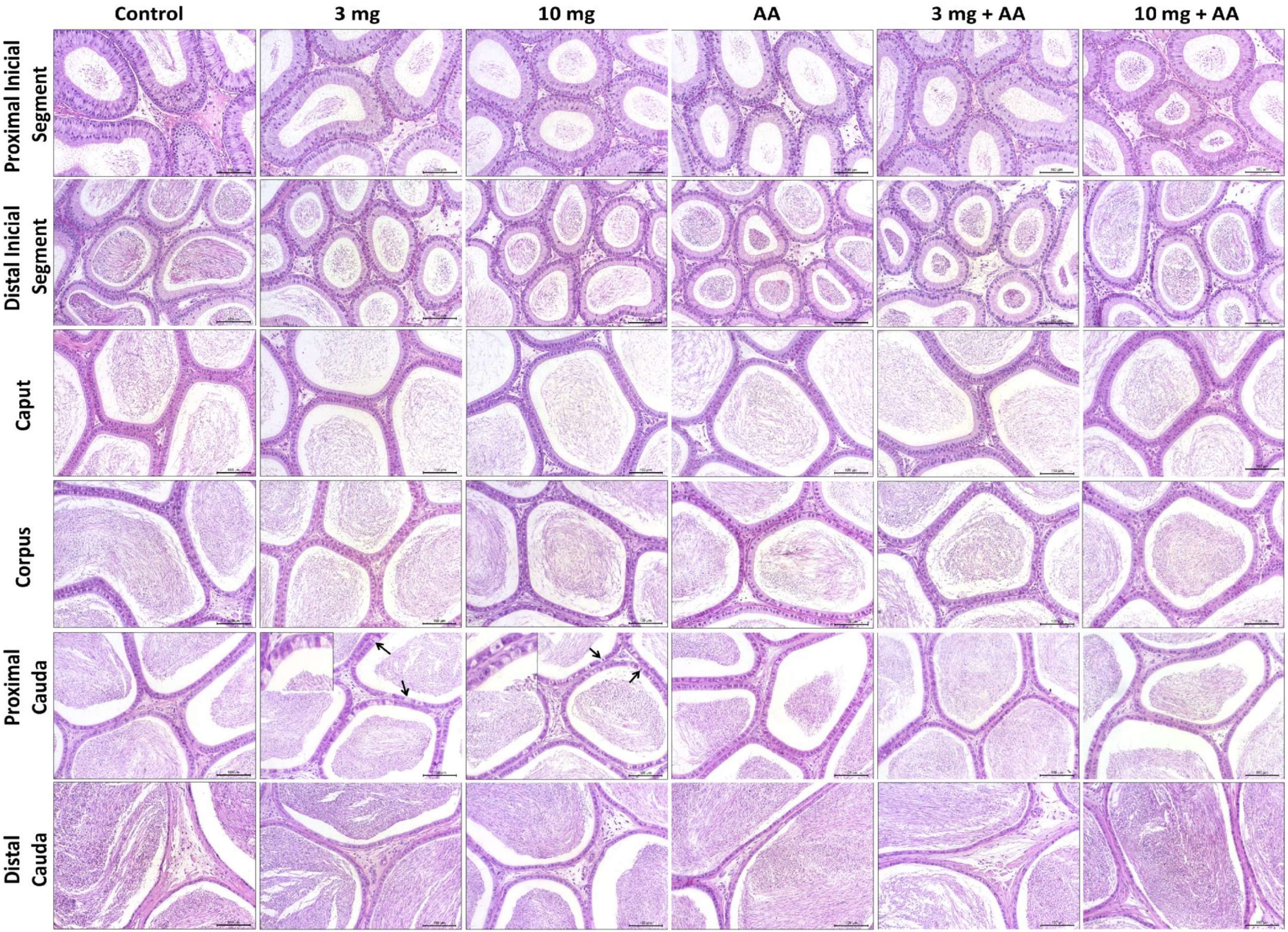

Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
Proximal Inicial Segment
Distal Inicial Segment
Caput
Corpus
Proximal Cauda
Distal Cauda

**Figure 4.**

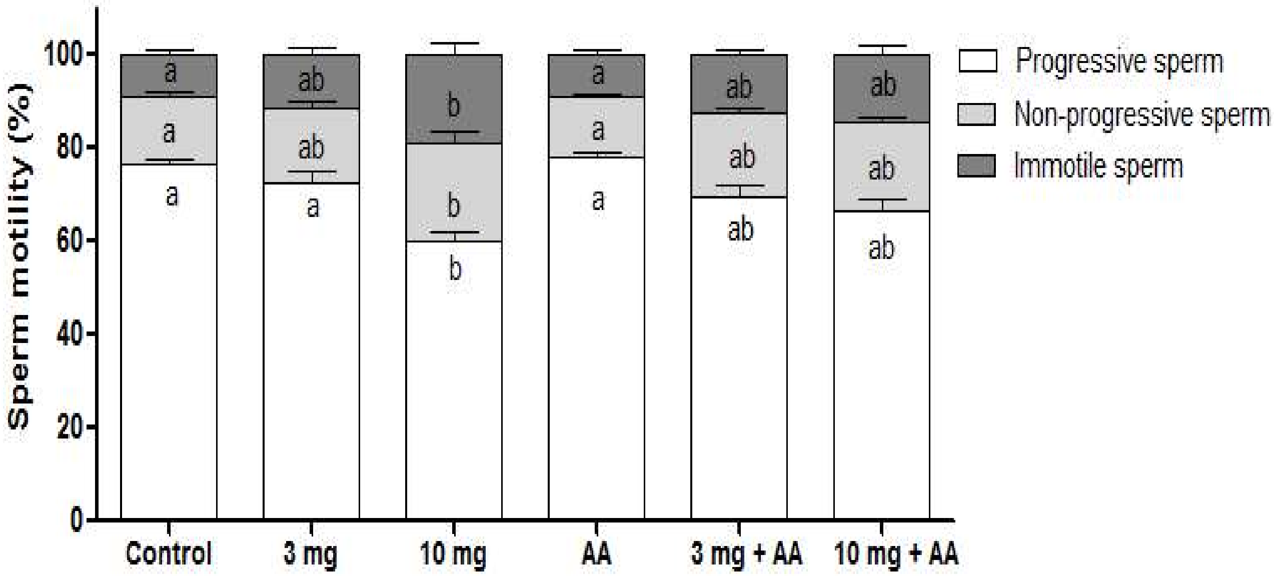

Sperm motility (%)
100
80
60
40
20
0
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
Progressive sperm
Non-progressive sperm
Immotile sperm
a
ab
b

**Figure 5.**

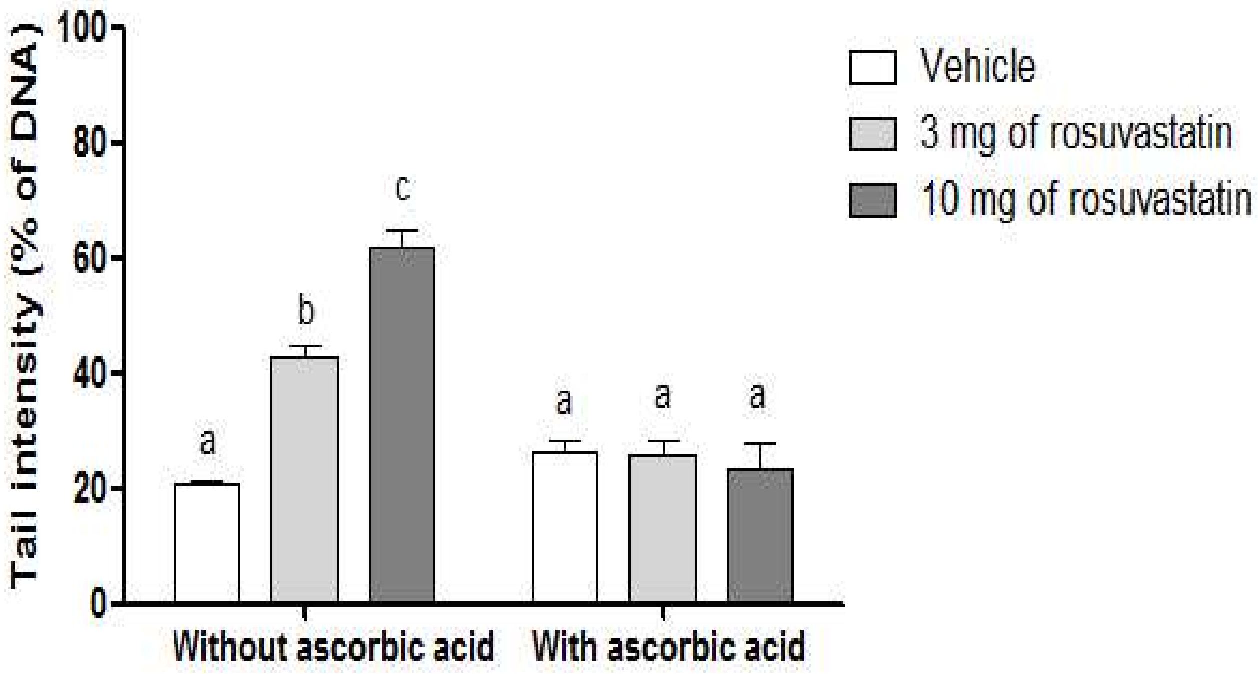
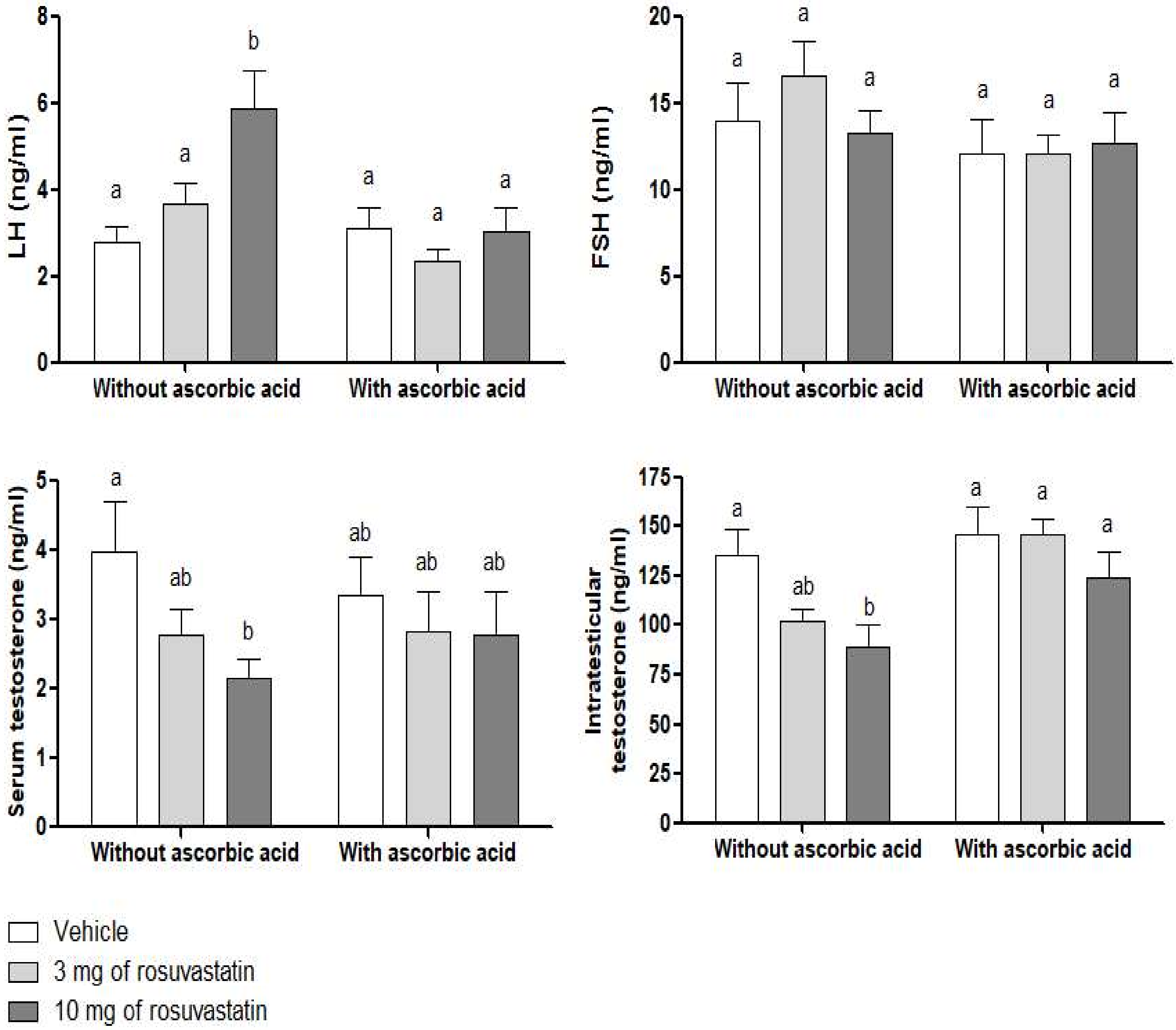
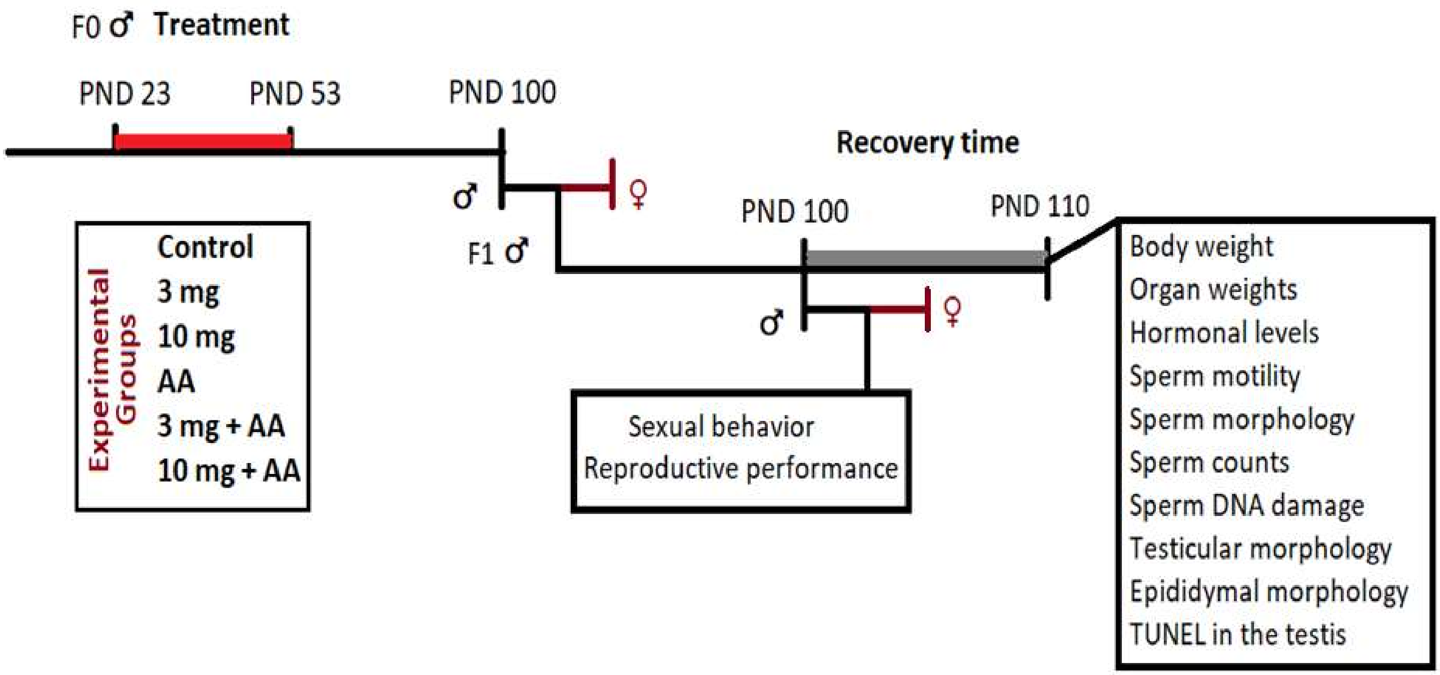

Tail intensity (% of DNA)
100
80
60
40
20
0
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin
a
b
c
a
a
a
Without ascorbic acid
With ascorbic acid

**Figure 6.**


LH (ng/ml)
8
6
4
2
0
a
a
b
a
a
a
Without ascorbic acid
With ascorbic acid
FSH (ng/ml)
20
15
10
5
0
a
a
a
a
a
a
Serum testosterone (ng/ml)
5
4
3
2
1
0
a
ab
b
ab
ab
ab
Intratesticular testosterone (ng/ml)
175
150
125
100
75
50
25
0
a
ab
b
a
a
a
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Supplementary Material**

**Figure 1.** Description of the experimental design.



# *Capítulo 5*

**Manuscrito V**

O quinto manuscrito é intitulado **"Reproductive outcomes in rat female offspring from male rats co-exposed to rosuvastatin and ascorbic acid during pre-puberty"** e será submetido na revista Journal of Toxicology and Environmental Health, Part A, Taylor & Francis, ISSN: 1528-7394. Fator de impacto: 2,731.

**Reproductive outcomes in rat female offspring from male rats co-exposed to rosuvastatin and ascorbic acid during pre-puberty**

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**Abstract**

Dyslipidemias are occurring earlier in different countries due to the increase of obesity, bad eating habits and sedentarism. Rosuvastatin reduces serum cholesterol; however, several studies have associated statin exposure with male reproduction impairment. Ascorbic acid (AA) is an antioxidant substance that plays a protective role in male reproductive system. Male rats were randomly divided into six experimental groups (n=10), which received saline solution 0.9%, 3 or 10 mg/Kg/day of rosuvastatin, 150 mg/day of AA or 3 or 10 mg/Kg/day of rosuvastatin associated with 150 mg/day of AA from post-natal day (PND) 23 until PND 53. On PND 100, males were mated with non-treated female rats to obtain the female pups. The day of vaginal opening and first estrus were assessed in the offspring. Two sets of females were euthanized on the first estrus after PND 42 and PND 75 to evaluate the histology of reproductive organs and hormone levels. A third set was used for sexual behavior and fertility test around PND 75. Female offspring from males exposed or co-exposed to the higher dose of statin showed a lower number of corpora lutea during puberty. On sexual maturity, the experimental group from males that were exposed to 3 mg showed lower uterine luminal epithelium area. Paternal exposure to rosuvastatin at pre-puberty delays ovarian development and diminishes uterine luminal epithelium in the female offspring, suggesting epigenetic changes provoked by statin. Otherwise, ascorbic acid co-administered to pre-pubertal males was able to ameliorate the reproductive damage in the rat female offspring at adulthood.

**Keywords:** Vitamin C; rosuvastatin; female offspring; reproduction; toxicology.

## 1. Introduction

Dyslipidemias have increased in the pediatric patients in consequence of the augmented prevalence of obesity (Jiménez and Ferre 2011) and have been defined as dysfunctions on lipid profile that includes increased LDL-cholesterol and VLDL-cholesterol, augmented triglycerides or reduced HDL-cholesterol concentrations in the blood (Kwiterovich 2008b; Kwiterovich 2008a; Jiménez and Ferre 2011).

Previous studies have reported that pediatric dyslipidemias may be linked to genetic factors such as familial hypercholesterolemia (Cook and Kavey 2011; Ross 2016) or associated with environmental factors like inappropriate life style, lack of physical exercises and bad eating habits (Izar, Fonseca, and Fonseca 2011; Ross 2016).

Among the lipid-lowering drugs, statins have highlighted due to their efficient reduction of total cholesterol in the blood (Tandon et al. 2005; Endres 2006; Istvan 2003). Statins decrease cholesterol concentrations by inhibiting the enzyme 3-hydroxy-3-methylglutharyl coenzyme A reductase (HMG-CoA reductase) (Istvan and Deisenhofer 2001; Jiménez and Ferre 2011) and preventing the conversion of HMG-CoA to mevalonate, thus reducing intermediate isoprenoids and cholesterol formation (Istvan 2003; Adam and Laufs 2008).

Rosuvastatin is the most recent statin available in the market used for lipid-lowering treatment and shows superior inhibitory effects on cholesterol biosynthesis in relation to the other statins (Holdgate, Ward, and McTaggart 2003; McTaggart 2003; Olsson, McTaggart, and Raza 2002). On the other hand, previous studies showed that rosuvastatin was capable to reduce serum testosterone concentrations (Hsieh and Huang 2016; Leite et al. 2014), interfere with male reproductive development (Leite et al. 2014; Leite et al. 2017a) and reduce sperm quality on sexual maturity (Leite et al. 2017b).

Several chemical agents, named as endocrine disruptors, may interfere with puberty timing and acquisition of reproductive capability when humans are exposed to them during critical periods, such as puberty (Stoker et al. 2000), and furthermore, they may provoke reproductive impairment at adulthood (Perobelli et al. 2012; Perobelli et al. 2013).

Recent studies have used different substances that may prevent oxidative stress or reduce the impairment provoked by the exposure to a drug (Corsetti et al. 2011; Sooriyaarachchi, Narendran, and Gailer 2012; Mukhopadhyay et al. 2013; Pandir, Kara, and Kara 2014), since various medications are important to treat diseases and still present non-benefit effects that are dispensable during the period of treatment (Sooriyaarachchi, Narendran, and Gailer 2012; Pandir, Kara, and Kara 2014).

Ascorbic acid is a necessary vitamin for many biological functions, such as α-tocopherol recycling and collagen biosynthesis (Sönmez, Turk, and Yuce 2005; Fernandes et al. 2011a; Fernandes et al. 2011b), besides to be essential for sperm integrity, fertility and on diminishing oxidative stress in the testis (Agarwal, Prabhakaran, and Said 2005; Eskenazi et al. 2005; Shrilatha and Muralidhara 2007; Fernandes et al. 2011).

Currently, many studies have shown that paternal exposure to a toxic compound may affect not only reproductive capability of the exposed animals but also may compromise reproduction of their male or female offspring (Favareto, de Toledo, and Kempinas 2011; Silva et al. 2016; Schagdarsurengin and Steger 2016; Zhao et al. 2015).

Considering the utilization of lipid-lowering medications for children and adolescents to decrease total cholesterol, to prevent cardiovascular diseases and the reproductive damage observed in rats following statin exposure, as previously reported, the present study aimed to evaluate the reproductive parameters on female offspring from rosuvastatin-exposed rats and assess the role of paternal supplementation with ascorbic acid on female offspring reproduction and fertility.

## 2. Material and Methods

### 2.1. Animals

#### 2.1.1. Obtainment of pregnant rats and reduction of litters

Male and female nonpathogenic free Wistar rats (45 days of age) were supplied from Central Biotherium of São Paulo State University (UNESP), Botucatu/SP and maintained in the Small Mammal Biotherium of the Morphology Department at the UNESP Biosciences Institute, Botucatu during the experiment.

Rats were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory grade pine shavings as bedding. Animals were maintained under controlled temperature (23 ± 1°C) and lighting conditions (12:12h photoperiod). The health status of the animals was monitored throughout the experiment. Standard rodent chow (Purina Labina, Agribrands do Brasil Ltda, Paulínia/SP, Brazil) and filtered tap water were provided *ad libitum*.

During adulthood, two nulliparous female rats (75 days of age) were mated with one male (90 days of age) during the dark phase of the lighting cycle and the day of sperm detection in the vaginal smear of female rats in estrus was considered gestational day 0 (GD 0). Pregnant and lactating female rats were maintained in individual cages.

After the birth of pups, their number per litter was culled to eight on postnatal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not considered into the experimental protocol and were posteriorly euthanized.

**2.1.2. Experimental design**

Male pups were randomly distributed into six experimental groups on PND 23 (n=10 per group, with one pup per litter for each group), that received vehicle (saline solution 0.9%), 3 or 10 mg/Kg/day of rosuvastatin diluted in saline solution 0.9%, supplementation with 150 mg/day of ascorbic acid or 150 mg/day of ascorbic acid associated with 3 or 10 mg/Kg/day of rosuvastatin. Ascorbic acid and rosuvastatin were purchased from a commercial pharmacy (Farmácia Botica Oficinal, Botucatu/Brazil). The drugs were administered orally by gavage since PND 23 until PND 53, following male pubertal assay of 31 days recommended by Environmental Protection Agency (EPA) (Stoker et al. 2000).

The available doses of rosuvastatin utilized by humans to reduce total cholesterol and LDL-cholesterol are between 5 and 40 mg/day (Vaughan and Gotto Jr 2004), thus the doses used in this study were based on body surface area correction from children available doses of rosuvastatin to pup rats equivalent doses (Reagan-Shaw, Nihal, and Ahmad 2008). The doses of ascorbic acid supplementation were based on previous studies (Fernandes et al. 2011a; Fernandes et al. 2011b).

Rats were maintained until PND 100 when were mated with nulliparous female rats to obtain their female offspring (generation F1). After the birth of pups, litters were reduced to eight on postnatal day (PND) 4 to balance male and female pups (4 each per litter). Litters with fewer than eight pups were not considered into the experiment and were subsequently euthanized.

Female offspring were evaluated in relation to puberty onset, ovarian and uterine histopathology and hormonal concentrations at the first estrus after PND 42 (one female per litter). Another female per litter was maintained until sexual maturity when sexual behavior test and reproductive performance and fertility test were assessed. The remaining rats were evaluated in relation to estrous cyclicity and then, were euthanized on the first estrus after PND 75 to obtain final body weight, reproductive and vital organ weights, hormonal concentrations, ovarian and uterine histopathology and histomorphometric evaluation in the ovaries and uterus (one female per litter). This experimental design is shown in Figure 1.

Animals were monitored in relation to the indications of distress, such as the ingestion of food and water and the presence of bristling hair during the whole experiment. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol number 589-CEUA).

**2.2. External physical sign of puberty onset**

Since PND 30, all female pups were assessed daily for complete vaginal opening from 8:00 to 10:00 a.m. After vaginal opening, the rats were weighed and evaluated in relation to the day of the first estrus through vaginal fluid cells content from 8:00 to 10:00 a.m., as previously described (Marcondes, Bianchi, and Tanno 2002). Ten microliters of saline solution 0.9% were inserted into the vagina and subsequently aspirated. Vaginal fluids were placed into slides and posteriorly analyzed under a light microscope (Zeiss, Axiostar Plus, Oberkochen, Germany) at x200 magnification. The estrus phase was characterized by the abundance of cornified vaginal epithelial cells.

**2.3. Estrous cyclicity**

On PND 60, one female per litter was evaluated daily until PND 75 in relation to the estrous cyclicity, using cells collected from vaginal fluid (as described above for the first estrus) to determine the estrous cycle phase. The presence and composition of the vaginal cells were used to characterize each phase: proestrus, predominance of nucleated epithelial cells; estrus: abundance of cornified epithelial cells; metaestrus, presence of nucleated and cornified epithelial cells and leukocytes; diestrus, predominance of leukocytes, followed by abundant vaginal mucus (Marcondes, Bianchi, and Tanno 2002). Data obtained during the fifteen days of analysis were used to estimate the total length of each phase of the cycle in days (proestrus, estrus, metaestrus and diestrus), the estrous cycle length and the number of cycles during the assessed period.

**2.4. Euthanasia of the females, body weight and organ weights**

Female offspring was weighed on the first estrus immediately after PND 42 or PND 75 (one female per litter in each period) and euthanized following narcosis by $CO_2$ asphyxiation and thereafter, blood was collected by inferior vena cava soon after confirming of estrus, between 9:00 and 11:30 a.m. Reproductive organs, such as ovaries, oviducts and uterus with fluid, from the rats, were collected and weighed. Vital organs that have an important role for toxicological parameters, such as kidney, adrenal, liver, thyroid, pituitary and brain were obtained and weighed.

**2.5. Serum hormonal concentrations**

Serum was obtained by centrifugation (2400 rpm, 20 minutes, 4ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal measurements. Progesterone, FSH and LH were determined by double-antibody radioimmunoassay. Plasma LH and FSH concentrations were determined using specific kits provided by the National Hormone and Peptide Program (Harbor-UCLA, USA). The primary antibodies for LH and FSH were anti-rat LH-S10 and FSH-S11 and the references were LH-RP3 and FSH-RP2, respectively. The lower limit of detection for LH was 0.04 ng/mL and for FSH, 0.2 ng/mL. The intra-assay coefficients of variation were 3.4% for LH and 3.0% for FSH. Serum concentrations of progesterone were determined using specific kits provided by MP Biomedicals (Orangeburgh, NY, USA). The intra-assay coefficient of variation was 3,6% and the lower limit of detection 0,02ng/mL. All samples were measured in the same assay to avoid the inter-assay errors.

**2.6. Histological procedures**

Uterus and left ovary were collected and fixed in Bouin's fluid, embedded in Paraplast® and sectioned in 4 µm (transversal sections of the ovary and middle region of the uterus). Sections were stained with hematoxylin and eosin (HE) to evaluate ovarian and uterine morphology under light microscopy. The evaluation was performed in a blind assay and the photomicrographs were obtained using scanned slides and the software Pannoramic Viewer in a personal computer.

Ovarian and uterine cross-sections were randomly chosen in three non-serial sections per animal obtained with a distance of 50 μm among them. Ovarian follicles were counted and classified based on the different stages of follicular development and morphology, and the number of granulosa cell layers (Borgeest et al. 2002; Talsness et al. 2005).

Ovarian follicles were classified, as follow: primordial and primary follicles (enumerated together), only a cuboidal or squamous granulosa cell layer; preantral follicles, presence of two to four granulosa cell layers without an antral space; antral follicles, presence of three or more granulosa cell layer and a defined antral space; and atretic follicles, characterized by the presence of pyknotic granulosa cells, disorganized granulosa cell layer, degenerating oocyte and/or detachment of basement membrane (Borgeest et al. 2002; Talsness et al. 2005).

The uterine morphological evaluation aimed to assess the histological appearance of each region of the organ: perimetrium, myometrium and endometrium (endometrial stroma, uterine glands and luminal uterine epithelium).

**2.7. Histomorphometric analyses**

The area of the left ovary was measured using scanned slides and the software Pannoramic Viewer in a personal computer to obtain the number of ovarian follicles and corpora lutea per area of the ovary ($mm^2$). Moreover, the area of all acidophilic corpora lutea in the left ovary from adult female rats were measured using the software Pannoramic Viewer, and thereafter, the mean area of corpora lutea was obtained per animal.

In the uterus, morphometric analyses were performed to measure the area of perimetrium, myometrium, endometrial stroma, luminal uterine epithelium and total and luminal area of the organ. Moreover, the number of uterine glands was also quantified.

**2.8. Evaluation of female sexual behavior**

On the first estrus after PND 75, one female rat per litter was used for the mating tests. The rats were maintained on an inverted 12h light–dark cycle, with food and water *ad libitum.* For the evaluation of female sexual behavior, sexually experienced males were allowed fifteen mounts on the female. Results were expressed as the lordosis quotient (LQ, number of lordosis/fifteen mounts x 100). All females were used only once during the sexual behavior test.

## 2.9. Fertility and reproductive performance

This analysis was carried following natural mating. In the case of rats that ejaculated during the evaluation of sexual behavior, couples stayed together for an additional four hours, allowing a greater number of ejaculations.

Every morning, males were separated from the females, and vaginal smears of each female were examined for the presence of sperm. The day when sperm was found in the vaginal smear was considered day 0 of gestation (GD 0). On the 20th day of gestation (GD 20), females were weighed and then, euthanized following narcosis by $CO_2$ asphyxiation and decapitation. After collection of the uterus and ovaries, the gravid uterus was weighed and the following parameters were quantified: corpora lutea, implantation sites, reabsorptions and live fetuses. Fetal and placental weights were also determined.

Based on these results, the following were determined: gestation rate = number of pregnant females/ number of inseminated females x 100; fertility potential (efficiency of implantation) = implantation sites/ corpora lutea x 100; rate of post-implantation loss = number of implantations - number of live fetuses/ number of implantations x 100; sex ratio = number of male fetuses/ number of female fetuses x 100.

## 2.10. Statistical analysis

The results from the different parameters were compared and analyzed among the experimental groups using two-way ANOVA, followed by Bonferroni's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed on GraphPad Prism (version 5.00).

## 3. Results

The description of main results of female offspring from male rats exposed to the different experimental treatments is shown in Figure 2.

### Puberty onset and hormonal concentrations

The age of vaginal opening and first estrus on female offspring were similar among the experimental groups (Figure 3). Furthermore, on PND 42 there was an increase in LH concentrations on female offspring from males exposed to the both doses of rosuvastatin in association with ascorbic acid, in comparison with controls (Figure 4). FSH and progesterone concentrations did not exhibit significant differences among the groups on PND 42 (Figure 4). During sexual maturity, hormonal concentrations of FSH, LH and progesterone were similar among the experimental groups (Figure 5).

**Histopathology, ovarian follicle and corpora lutea quantification and uterine morphometries**

Ovarian histopathological assessment on the first estrus after PND 42 showed a lower number of corpora lutea per area of the organ in the female offspring from males exposed to the higher dose of rosuvastatin alone or in association with ascorbic acid when compared to the controls (Figures 6 and 7). On the other hand, follicle quantification per area of the ovary was similar among the groups at the same period (Figure 6).

There was an augmentation in the area of corpora lutea on sexual maturity, with no differences in the number of these endocrine structures in the female offspring from males exposed to the association of rosuvastatin and ascorbic acid, when compared to the controls (Figures 7 and 8). The number of follicles per area of ovary did not show significant differences among the experimental groups (Figure 8).

The uterine morphometry on PND 42 showed a similar number of endometrial glands and no statistical differences for total and luminal areas of the organ among the experimental groups (Figure 9). Perimetrium, myometrium, endometrial stroma and luminal uterine epithelial area were similar among the groups (Figure 9). Additionally, uterine tissue exhibited similar morphology among the experimental groups (Figure 10).

Uterine luminal epithelial area was lower at sexual maturity in the offspring from males treated with the lower dose of rosuvastatin, when compared to the controls (Figures 10 and 11). Total and luminal areas of the organ and perimetrium, myometrium and endometrial stroma areas showed no differences among the experimental groups (Figure 11). Moreover, the number of uterine glands were similar among the groups (Figure 11).

**Estrous cycle, sexual behavior testing and reproductive performance**

Estrous cycle assessment showed similar length and number of cycles during the evaluated period, as well as, comparable duration of each phase of the cycle among the groups

(Table 1). In addition, sexual behavior testing and reproductive performance and fertility showed no differences among the groups (Figure 12 and Table 2).

**Body weight and organ weights**

Final body weight exhibited no differences among the experimental groups on the first estrus after PND 42 and PND 75 (Table 3 and 4). Reproductive and vital organ weights did not show significant differences among the groups on the first estrus after PND 42 (Table 3 and 5). On the other hand, ovarian weight was augmented at adulthood in the female offspring from males co-exposed to 10 mg of rosuvastatin and ascorbic acid, in comparison to the other groups (Table 4). The remaining reproductive and vital organ weights did not show significant differences among the groups on PND 75 (Table 4 and 6).

## 4. Discussion

The study is based on the utilization of lowering-lipid medications by children and adolescents that need the drug to improve their lipid profile by reducing total cholesterol and triglycerides levels or to prevent cardiovascular disease risk. The use of ascorbic acid showed to be useful to alleviate the reproductive impairment in males provoked by rosuvastatin exposure during prepuberty (Leite et al. 2017a; Leite et al. 2017b). Although this work was performed using non-obese juvenile Wistar rats, it simulates the human exposure, since it has also been reported increased prevalence of atherosclerosis in children and adolescents (Ross 2016; Wiegman et al. 2015) and in addition, more people are taking benefits from the pleiotropic effects of statins (Ludman et al. 2009; Tandon et al. 2005).

Considering the lack of information about the reproductive effects for the female offspring from males exposed to lowering-lipid drugs, the present study aimed to assess the reproductive development of female offspring from male rats exposed to rosuvastatin during prepuberty, besides the possible protective role of paternal supplementation with ascorbic acid for female offspring reproductive development.

Puberty onset is the beginning of cyclical events that direct the anatomy and function of female reproductive system (Andersson et al. 2013). Increased FSH levels secreted by pituitary leads to the beginning of ovarian maturation, which is accompanied by augmented levels of estrogen (Guerra et al. 2017; Andersson et al. 2013). Furthermore, the age of vaginal opening and first estrus occurrence has been considered as an important external sign of puberty installation and is highly correlated with augmented estrogen levels (U.S. Environmental Protection Agency 1996). In this work, the age of puberty onset were similar among the groups, although the offspring from males exposed to the higher dose of rosuvastatin alone or in association with ascorbic acid have shown delayed ovarian maturation.

Estrogen concentrations increase with follicular growth, which is stimulated by FSH synthetized by anterior pituitary (Shaikh 1971; Andersson et al. 2013). Augmented estrogen levels stimulate LH surge and then, ovulation and luteinization occurs and corpora lutea secretes progesterone (Andersson et al. 2013). In this study, during pubertal period FSH and progesterone levels did not exhibit differences among the groups; however, LH levels were augmented in offspring from the co-exposed groups, indicating that the interaction of these substances is able to interfere genetically (Leite et al. 2017b) or maybe epigenetically with paternal sperm and consequently increase LH on pubertal female offspring. Additional studies are necessary to clarify the molecular mechanisms involved in these findings. At adulthood, FSH, LH and progesterone measurements showed to be similar among the groups.

Rat ovarian maturation is a result from a cascade of neuroendocrine events that include the establishment of pulsatile LH levels and increased prolactin and GH concentrations at thirty days of age approximately, thus culminating on puberty installation in female rats (Andrews and Ojeda 1981; Westwood 2008). The number of corpora lutea increases with age in prepubertal female rats and it is considered as a parameter for interpretation of ovarian development and maturation (Picut et al. 2014a; Picut et al. 2014b). Furthermore, it is known that during puberty the rate of necrotic ova in the medulla decreases (Picut et al. 2014a; Picut et al. 2014b). Pubertal female rats from males exposed to the higher dose of statin alone or in association with ascorbic acid showed lower number of corpora lutea, suggesting delayed ovarian maturation in these groups.

During pubertal period, increased LH concentrations on estrus in the offspring from male rats co-exposed to the higher dose of statin may be a consequence of lower ovulatory rate in pubertal females, suggesting ovarian hypofunction. Diminished number of ovulated oocytes leads to lower number of corpora lutea and reduced efficiency of negative feedback to inhibit LH releasing, thus producing higher basal LH. This deficiency appears to occur only at pubertal period, because LH levels and corpora lutea number become similar among the groups at adulthood.

During pubertal period, uterus shows a higher luminal uterine epithelium, a columnar epithelium, besides a common degeneration in epithelial luminal cells and the presence of leukocyte infiltration on endometrial stroma (Picut et al. 2014b). Pubertal uterine tissues showed a similar morphological aspect among the experimental groups in this study. Additionally, uterine morphometries showed no differences in relation to the area of each region of the organ.

Hormonally mediated effects on female reproductive system may occur in different ways, such as disrupting estrous cycle, organ weights changes and histopathological alterations (Andersson et al. 2013). Uterine epithelium is a tissue highly responsive to estradiol concentrations, since augmented estradiol concentrations leads to increased height of the epithelium and thicker endometrial stroma (Uslu et al. 2013) and lower estradiol concentrations leads to epithelial atrophy (Andersson et al. 2013). In this work, female offspring from male rats exposed to the lower dose of statin exhibited a lower columnar uterine epithelium and diminished area of luminal epithelial uterine compartment. Although we do not have measured estradiol concentrations due to lack of reliable kits for radioimmunoassay nowadays, it is possible to correlate this histopathological finding with a possible reduction on estradiol concentrations in this group.

Previous *in vitro* studies have reported that theca-interstitial cells exposed to simvastatin, another statin, showed decreased isoprenylation and expression of CYP17A1, thus leading to diminished androgen production (Ortega et al. 2012; Ortega et al. 2014). In view of lower concentration of androgens that are synthesizing by theca-interstitial cells due to statin exposure, granulosa cells may be producing low estrogen levels because they are receiving lower concentrations of substrates to be aromatized to estradiol.

In the adult rat ovary is found all stages of follicles at all phases of estrous cycles, as well as corpora lutea, however corpus luteum shows different morphology depending on the phase of estrous cycle (Westwood 2008). During estrus phase, the number of follicles and corpora lutea per area of ovary were similar among the groups. On the other hand, female offspring from the group co-exposed to 10 mg of rosuvastatin showed increased area of corpora lutea, without an augmentation on serum progesterone concentrations and in the number of these endocrine structures. Indeed, progesterone secretion by corpora lutea on estrus is basal and they only show their ability to secrete progesterone on metaestrus, when progesterone surge occurs. Additionally, augmented corpora lutea area may be associated with increased prolactin levels in females.

Final body weight evaluation supplies substantial information about the health status of the animals (Clegg, Perreault, and Klinefelter 2001). Moreover, reproductive organ weights provides information about the reproductive system (Clegg, Perreault, and Klinefelter 2001) and vital organ weights may provide a general systemic health condition of the animals. This study reports that there was an augmentation of ovarian weight in the female offspring from males co-exposed to 10 mg of statin and ascorbic acid. This result is reinforced by larger corpora lutea area exhibited in this experimental group. Body weight and the remaining organ weights were similar among the groups.

The interference with reproductive function of female rat is often expressed as a disturbance in the length of estrous cycle or histopathological changes on reproductive organs (Westwood 2008), thus estrous cycle monitoring provides information about female reproductive status. This study shows no changes in estrous cycle dynamics in female offspring from male rats exposed to rosuvastatin and/or ascorbic acid.

The lordotic posture is the main sign of female rat receptivity (Pfaff and Sakuma 1979; Veening, Coolen, and Gerrits 2013), which is only performed under adequate hormonal conditions (Veening, Coolen, and Gerrits 2013; Pfaff and Sakuma 1979). Furthermore, lordosis is a graded response and may be performed in different intensities depending on female hormonal status, environmental conditions or male rat sexual behavior (Pattij et al. 2005; Veening, Coolen, and Gerrits 2013). Lordosis quotient did not exhibit significant differences among the experimental groups in this study, thus insinuating that paternal exposure to rosuvastatin or even ascorbic acid does not change sexual behavior in the female offspring.

It is known that epidemiological data about post-implantation loss rates in non-treated laboratory rodents is about 10% or lower (Jauniaux, Poston, and Burton 2006). In this study, reproductive performance did not show differences among the groups and post-implantation loss rate was about 10% or less than this, thus suggesting that paternal treatments with statin and ascorbic acid does not affect these parameters.

Paternal or maternal exposure to hazardous substances may modify gamete epigenome and affect the health of the children (Schagdarsurengin and Steger 2016). Sperm epigenetic modifications may affect reproduction leading to spermatogenesis failure, compromising embryo development and producing long-term effects during offspring lifetime (Stuppia et al. 2015).

Changes on DNA methylation and histone modifications including acetylation and methylation are the main characterized epigenetic changes (Rajender et al., 2011). Various studies have been reported that sperm epigenetic changes may interfere with embryo development and causes diseases in the offspring, such as reproductive dysfunctions (Silva et al. 2016; Favareto, de Toledo, and Kempinas 2011; Schagdarsurengin and Steger 2016; Stuppia et al. 2015; Ly et al. 2017).

Previous study has reported that augmented DNA damage generated by oxidative stress is highly related to aberrant global methylation (Rajender et al., 2011). Contrary to this, antioxidants diminished the amount of DNA fragmentation and oxidative stress, as well as increased global hypomethylation (Rajender et al., 2011).

It is well established that histone acetylation induces local chromatin expansion and activation of gene transcription (Schagdarsurengin and Steger, 2016). Previous study showed that statins reduce acetylation of histone H3 and H4 and phosphorylation of H3 (Arrigoni et al., 2017). Ascorbate, intracellular form of ascorbic acid, regulates DNA demethylation due to its role as essential cofactor for Ten-eleven translocation (TET) dioxygenases. Furthermore, ascorbate regulates histone demethylation because acts as a crucial cofactor for Jumonji C-domain-containing histone demethylases (Young et al., 2015).

This is a new report about the reproductive effects for female offspring from male rats exposed to statins and/or supplemented with ascorbic acid. There are few studies concerning statin exposure and reproductive effects and none reporting intergenerational reproductive effects affecting female offspring. The results suggest epigenetic effects, besides increased paternal sperm DNA fragmentation associated with rosuvastatin exposure, as previously reported (Leite et al. 2017b). Further studies are necessary to elucidate the molecular mechanisms involved on the promotion of female reproductive damage mediated by paternal sperm changes.

## 5. Conclusions

In summary, paternal exposure to the higher dose of rosuvastatin in association or not with ascorbic acid delayed ovarian development, as shown by the lower number of corpora lutea, although without affecting ovarian function at adulthood. In addition, female offspring from males exposed to the lower dose of statin showed lower columnar uterine epithelium, insinuating reduced estradiol concentration. The data suggest important genetic and/or epigenetic changes in paternal sperm mediated by statin exposure. Ascorbic acid, when administered to males, is capable to protect paternal sperm somehow, thus reducing the reproductive impairment on female offspring from males exposed to statin.

## 6. Conflicts of interest

The authors declare that there are no conflicts of interest.

## 7. Acknowledgments

The authors are grateful to to José Eduardo Bozano, from the Department of Morphology, Institute of Biosciences, UNESP, Botucatu/SP – Brazil, for his excellent technical assistance and to Dr. Ruither de Oliveira Gomes Carolino, from the Department of Morphology, Stomatology and Physiology, School of Dentistry, University of São Paulo – USP, Ribeirão Preto/SP, Brazil, for his contributions to hormonal measurements.

## 8. Funding

This research was financially supported by São Paulo Research Foundation (FAPESP) (Grants #013/22495-1 and #014/13659-3), National Council for Scientific and Technological Development (CNPq) (Grant 308842/2013-8) and Coordination for the Improvement of Higher Education Personnel (CAPES).

## 9. References

Adam, O., and Laufs, U. 2008. Antioxidative Effects of Statins. *Arch. Toxicol.* 82: 885–92.

Agarwal, A., Prabhakaran, S. A., and Said T. M. 2005. Prevention of Oxidative Stress Injury to Sperm. *J. Androl.* 26: 654–60.

Andersson, H., Rehm, S., Stanislaus, D., and Charles E. W. 2013. Scientific and Regulatory Policy Committee (SRPC) Paper : Assessment of Circulating Hormones in Nonclinical Toxicity Studies. *Toxicol. Pathol.* 41: 921–34.

Andrews, W. W., and Ojeda, S. R. 1981. Secretory Profile in Conscious, Free-Moving Female

Rats during the Time of Puberty. *Endocrinology* 109: 2032–39.

Borgeest, C., Symonds, D., Mayer, L. P., Hoyer P. B., and Flaws J. A. 2002. Methoxychlor May Cause Ovarian Follicular Atresia and Proliferation of the Ovarian Epithelium in the Mouse. *Toxicol. Sci.* 68: 473–78.

Clegg, E. D., Perreault, S.D., and Klinefelter, G.R. 2001. Assessment of Male Reproductive Toxicology. In *Principles and Methods of Toxicology*, 4th ed., ed. A.W Hayes, 1263–99. Philadelphia, PA: Taylor & Francis.

Cook, S., and Kavey, R. E. W. 2011. Dyslipidemia and Pediatric Obesity. *Pediatr. Clin. North Am*. 58: 1363–73.

Corsetti, G., Stacchiotti, A., Tedesco, L., D'Antona, G., Pasini, E., Dioguardi, F. S., Nisoli, E., and Rezzani, R. 2011. Essential Amino Acid Supplementation Decreases Liver Damage Induced by Chronic Ethanol Consumption in Rats. *Int. J. Immunopathol. Pharmacol*. 24: 611–19.

Endres, M. 2006. Statins: Potential New Indications in Inflammatory Conditions. *Atherosclerosis Suppl*. 7: 31–35.

Eskenazi, B., Kidd, S. A., Marks, A. R., Sloter, E., Block, G., and Wyrobek, A. J. 2005.

Antioxidant Intake Is Associated with Semen Quality in Healthy Men. *Hum. Reprod.* 20: 1006–12.

Favareto, A. P. A., de Toledo, F. C., and Kempinas, W. D. G. 2011. Paternal Treatment with Cisplatin Impairs Reproduction of Adult Male Offspring in Rats. *Reprod. Toxicol.* 32: 425–33.

Fernandes, G. S. A., Fernandez, C. D. B., Campos, K. E., Damasceno, D. C., Anselmo-Franci, J. A., and Kempinas, W. D. G. 2011a. Vitamin C Partially Attenuates Male Reproductive Deficits in Hyperglycemic Rats. *Reprod. Biol. Endocrinol.* 100: 1–9.

Fernandes, G. S. A., Gerardin, D. C. C., Assumpção, T. A., Campos, K. E., Damasceno, D. C., Pereira, O. C. M., and Kempinas, W. D. G. 2011b. Can Vitamins C and E Restore the Androgen Level and Hypersensitivity of the Vas Deferens in Hyperglycemic Rats? *Pharmacol. Rep.* 63: 983–91.

Guerra, M. T., Sanabria M., Cagliarani, S. V., Leite, G. A. A., Borges, C. S., Kempinas, W. G. 2017. Long-Term Effects of in Utero and Lactational Exposure to Butyl Paraben in Female Rats. *Environ. Toxicol.* 32: 776–88.

Holdgate, G. A., Ward, W. H. J., and McTaggart, F. 2003. Molecular Mechanism for Inhibition of 3-Hydroxy-3-Methylglutaryl CoA (HMG-CoA) Reductase by Rosuvastatin. *Biochem. Soc. Trans.* 31: 528–31.

Hsieh, C. J., and Huang, B. 2016. Rosuvastatin Decreases Testosterone Levels but Not Sexual Function in Men with Type 2 Diabetes. *Diabetes Res Clin. Pract.* 120: 81–88.

Istvan, E. S. 2003. Statin Inhibition of HMG-CoA Reductase: A 3-Dimensional View. *Atherosclerosis Suppl.* 4: 3–8.

Istvan, E. S., and Deisenhofer, J. 2001. Structural Mechanism for Statin Inhibition of HMG-CoA Reductase. *Science.* 292: 1160–64.

Izar, M.C. D. O., Fonseca M. I. H., and Fonseca F. A. H. 2011. Dyslipidemias. *Rev. Bras. Med.* 68: 59–73.

Jauniaux, E. , Poston, L., and Burton, G. J. 2006. Placental-Related Diseases of Pregnancy : Involvement of Oxidative Stress and Implications in Human Evolution. *Hum. Reprod. Update.* 12: 747–55.

Jiménez, E. G., and Ferre, J. A. 2011. Estatinas : Características Y Efectos Sobre El Control Lipídico En El Niño Y Adolescente Obeso. *Rev. Clin. Med. Fam.* 4: 69–75.

Kwiterovich, P. O. 2008a. Clinical and Laboratory Assessment of Cardiovascular Risk in Children: Guidelines for Screening, Evaluation, and Treatment. *J. Clin. Lipidol.* 2: 248–66.

Kwiterovich, P. O. 2008b. Recognition and Management of Dyslipidemia in Children and Adolescents. *J. Clin. Endocrinol. Metab.* 93: 4200–09.

Leite, G. A. A., Figueiredo T. M., Sanabria M., Dias, A. F. M. G., Silva, P. V., Martins-Junior, A. C., Barbosa-Junior, F., and Kempinas, W. D. G. 2017a. Ascorbic Acid Supplementation Partially Prevents the Delayed Reproductive Development in Juvenile Male Rats Exposed to Rosuvastatin since Prepuberty. *Reproductive Toxicology*. (*in press*)

Leite, G. A. A., Figueiredo, T. M., Pacheco, T. L., Sanabria M., Silva, P. V., Fernandes F. H., and Kempinas, W. D. G. 2017. Vitamin C Partially Prevents Reproductive Damage in Adult Male Rats Exposed to Rosuvastatin during Prepuberty. *Food and Chemical Toxicology*. (*in press*)

Leite, G. A. A., Rosa, J. L., Sanabria M., Cavariani M. M., Anselmo-Franci, J. A., Pinheiro, P. F. F., and Kempinas W. D. G.. 2014. Delayed Reproductive Development in Pubertal Male Rats Exposed to the Hypolipemiant Agent Rosuvastatin since Prepuberty. *Reprod. Toxicol.*

44: 93–103.

Ludman, A., Venugopal, V., Yellon, D. M., and Hausenloy, D. J. 2009. Statins and Cardioprotection - More than Just Lipid Lowering? *Pharmacol. Ther.* 122: 30–43.

Ly, L., Chan, D., Aarabi, M., Landry, M., Behan, N. A., Macfarlane, A. J., and Trasler, J. 2017. Intergenerational Impact of Paternal Lifetime Exposures to Both Folic Acid de Fi Ciency and Supplementation on Reproductive Outcomes and Imprinted Gene Methylation. *Mol. Hum. Reprod.* 1: 1–17.

Marcondes, F. K., Bianchi, F. J., and Tanno, A. P. 2002. Determination of The Estrous Cycle Phases of Rats: Some Helpful Considerations. *Braz. J. Biol.* 62: 609–14.

McTaggart, F. 2003. Comparative Pharmacology of Rosuvastatin. *Atherosclerosis Suppl.* 4: 9–14.

Mukhopadhyay, P. K., Dey, A., Mukherjee, S., and Pradhan, N. K. 2013. The Effect of Coadministration of Alpha-Tocopherol and Ascorbic Acid on Arsenic Trioxide-Induced Testicular Toxicity in Adult Rats. *J. Basic Clin. Physiol. Pharmacol.* 24: 245–53.

Olsson, A. G., McTaggart, F., and Raza, A. 2002. Rosuvastatin: A Highly Effective New HMG-CoA Reductase Inhibitor. *Cardiovasc. Drug Rev.* 20: 303–28.

Ortega, I., Cress, A. B., Wong, D. H, Villanueva, J. A., Sokalska, A., Moeller, B. C., Stanley, S. D., and Duleba, A. J. 2012. Simvastatin Reduces Steroidogenesis by Inhibiting Cyp17a1 Gene Expression in Rat Ovarian Theca-Interstitial Cells. *Biol. Reprod.* 86: 1–9.

Ortega, I., Villanueva J. A., Wong D. H., Cress A. B., Sokalska, A., Stanley, S. D., and Duleba, A. J. 2014. Resveratrol Potentiates Effects of Simvastatin on Inhibition of Rat Ovarian Theca-Interstitial Cells Steroidogenesis. *J. Ovarian Res.* 21: 1–8.

Pandir, D., Kara, O., and Kara, M. 2014. Protective Effect of Bilberry (Vaccinium Myrtillus L.) on Cisplatin Induced Ovarian Damage in Rat. *Cytotechnology.* 66: 677–85.

Pattij, T., De Jong, T. R., Uitterdijk, A., Waldinger, M. D., Veening, J. G., Cools, A. R., Van Der Graaf, P. H., and Olivier, B. 2005. Individual Differences in Male Rat Ejaculatory Behaviour: Searching for Models to Study Ejaculation Disorders. *Eur. J. Neurosci.* 22: 724–34.

Perobelli, J. E., Alves, T. R., de Toledo, F. C., Fernandez, C. D. B., Anselmo-Franci, J. A., Klinefelter, G. R., and Kempinas, W. D. G. 2012. Impairment on Sperm Quality and Fertility of Adult Rats after Antiandrogen Exposure during Prepuberty. *Reprod. Toxicol.* 33:

308–15.

Perobelli, J. E., Patrão, M. T. C. C., Fernandez, C. D. B., Sanabria, M., Klinefelter, G. R., Avellar, M. C. W., and Kempinas, W. D. G. 2013. Androgen Deprivation from Pre-Puberty to Peripuberty Interferes in Proteins Expression in Pubertal and Adult Rat Epididymis. *Reprod. Toxicol.* 38: 65–71.

Pfaff, D. W., and Sakuma, Y. 1979. Facilitation of the Lordosis Reflex of Female Rats from the Ventromedial Nucleus of the Hypothalamus. *J. Physiol.* 288: 189–202.

Picut, C. A., Dixon D., Simons M. L., Stump D. G., Parker, G. A., and Remick, A. K. 2014. Postnatal Ovary Development in the Rat : Morphologic Study and Correlation of Morphology to Neuroendocrine Parameters. *Toxicol. Pathol*. 43: 343–53.

Picut, C. A., Remick A. K., Asakawa, M. G., Simons, M. L., and Parker, G. A. 2014. Histologic Features of Prepubertal and Pubertal Reproductive Development in Female Sprague-Dawley Rats. *Toxicol. Pathol.* 42: 403–13.

Reagan-Shaw, S., Nihal, M., and Ahmad, N. 2008. Dose Translation from Animal to Human Studies Revisited. *FASEB J.* 22: 659–61.

Ross, J. L. 2016. Statins in the Management of Pediatric Dyslipidemia. *J. Pediatr. Nurs.* 31: 723–35.

Schagdarsurengin, U., and Steger, K. 2016. Epigenetics in Male Reproduction: Effect of Paternal Diet on Sperm Quality and Offspring Health. *Nat. Rev. Urol.* 13: 584–95.

Shaikh, A. A. 1971. Estrone and Estradiol Levels in the Ovarian Venous Blood from Rats during the Estrous Cycle and Pregnancy. *Biol. Reprod.* 5: 297–307.

Shrilatha, B., and Muralidhara. 2007. Early Oxidative Stress in Testis and Epididymal Sperm in Streptozotocin-Induced Diabetic Mice: Its Progression and Genotoxic Consequences. *Reprod. Toxicol.* 23: 578–87.

Silva, P. V., Silva, R. F., Borges, C. S., Cavariani, M. M., Almeida-Francia, C. C. D., Barbosa-Junior, F., and Kempinas, W. D. G. 2016. Sexual Differentiation and Reproductive Development of Female Rat Offspring after Paternal Exposure to the Anti-Tumor Pharmaceutical Cisplatin. *Reprod. Toxicol.* 60: 112–22.

Sönmez, M., Turk, G., and Yuce, A. 2005. The Effect of Ascorbic Acid Supplementation on Sperm Quality, Lipid Peroxidation and Testosterone Levels of Male Wistar Rats. *Theriogenology.* 63: 2063–72.

Sooriyaarachchi, M., Narendran, A., and Gailer, J. 2012. The Effect of Sodium Thiosulfate on the Metabolism of Cis-Platin in Human Plasma in Vitro. *Metallomics.* 4: 960–67.

Stoker, T. E., Laws, S. C., Guidici, D. L., and Cooper, R. L. 2000. The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function. *Toxicol. Sci.* 58: 50–59.

Stuppia, L., Franzago, M., Ballerini, P., Gatta, V., and Antonucci, I. 2015. Epigenetics and Male Reproduction : The Consequences of Paternal Lifestyle on Fertility , Embryo Development , and Children Lifetime Health. *Clin. Epigenetics.* 7: 1–15.

Talsness, C. E., Shakibaei, M., Kuriyama, S. N., Grande, S. W., Sterner-Kock, A., Schnitker, P., Souza, C., Grote, K., and Chahoud, I. 2005. Ultrastructural Changes Observed in Rat Ovaries Following in Utero and Lactational Exposure to Low Doses of a Polybrominated Flame Retardant. *Toxicol Lett.* 157: 189–202.

Tandon, V., Bano, G., Khajuria, V., Parihar, A., and Gupta, S. 2005. Pleiotropic Effects of Statins. *Indian J. Pharmacol.* 37: 77–85.

U.S. Environmental Protection Agency. 1996. Guidelines for Reproductive Toxicity Risk Assessment. *E. P. A.* 61: 56274-322.

Uslu, U, Sandal, S., Cumbul, A., Yildiz, S., Aydin, M., and Yilmaz, B. 2013. Evaluation of Estrogenic Effects of Polychlorinated Biphenyls and Organochlorinated Pesticides Using Immature Rat Uterotrophic Assay. *Hum. Exp. Toxicol.* 32: 476–82.

Vaughan, C. J., and Gotto Jr, A. M. 2004. Update on Statins: 2003. *Circulation.* 110: 886–92.

Veening, J. G., Coolen, L. M., and Gerrits, P. O. 2013. Neural Mechanisms of Female Sexual Behavior in the Rat: Comparison with Male Ejaculatory Control. *Pharmacol. Biochem. Behav.* 121: 16–30.

Westwood, F. R. 2008. Toxicologic Pathology The Female Rat Reproductive Cycle : A Practical Histological Guide to Staging. *Toxicol. Pathol.* 36: 375–84.

Wiegman, A., Gidding, S. S., Watts, G. F., Chapman, M. J., Ginsberg, H. N., Cuchel, M., Ose, L., et al. 2015. Familial Hypercholesterolaemia in Children and Adolescents: Gaining Decades of Life by Optimizing Detection and Treatment. *Eur. Heart J.* 36: 2425–37.

Zhao, X., Cheng, Z., Zhu, Y., Li, S., Zhang, L., and Luo, Y. 2015. Effects of Paternal Cadmium Exposure on the Sperm Quality of Male Rats and the Neurobehavioral System of Their Offspring. *Exp. Ther. Med.* 10: 2356–60.

**Figure captions**

**Figure 1.** Description of the experimental design.

**Figure 2.** Description of the main results of female offspring from male rats exposed to the different experimental treatments.

**Figure 3.** Age of vaginal opening and first estrus in the rat female offspring. Values expressed as mean ± standard error of mean (SEM). Two-way ANOVA followed by Bonferroni's test. $p > 0.05$.

**Figure 4.** Serum hormonal concentrations (ng/ml) in rat female offspring at 42 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni’s test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 5.** Serum hormonal concentrations (ng/ml) in rat female offspring at 75 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni’s test. $p > 0.05$.

**Figure 6.** Follicle and corpora lutea quantification in the pubertal ovary of rat female offspring from experimental groups at 42 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni’s test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 7.** Photomicrography of ovarian sections in the female offspring from experimental groups on postnatal day (PND) 42 and 75. Observe the acidophilic corpora lutea (asterisks) in lower number in the ovaries of female offspring from males exposed and co-exposed to the dose of 10 mg of rosuvastatin. Note the presence of larger corpora lutea (arrows) in the ovary of female offspring from the co-exposed group to the higher dose of statin. Hematoxylin and Eosin (HE). Scale bar = 1000µm.

**Figure 8.** Follicle and corpora lutea quantification and corpora lutea area in the adult ovary of rat female offspring from experimental groups at 75 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni’s test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 9.** Morphometries in the pubertal uterus of female rat offspring from the experimental groups at 42 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni’s test. $p > 0.05$.

**Figure 10.** Photomicrography of uterine sections in the female offspring from experimental groups on PND 42 and 75. Observe the lower uterine luminal epithelium height (arrows) in the uterus of female offspring from males exposed to the dose of 3 mg of rosuvastatin. Hematoxylin and Eosin (HE). Scale bar = 1000µm or 50µm.

**Figure 11.** Morphometries in the adult uterus of female rat offspring from the experimental groups at 75 days of age. Values expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni's test. $p < 0.05$. Different letters indicate statistically significant differences among the groups.

**Figure 12.** Lordosis quotient in the female rat offspring from the experimental groups on PND 75. Values expressed as median and interquartile intervals. Two-way ANOVA followed by Bonferroni's test. $p > 0.05$.

**Table 1.** Estrous cyclicity assessment in the female offspring from experimental groups from post-natal day (PND) 60 until PND 75.

| | Experimental Groups (n= 9 or 10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| [1]Estrous cycle length (days) | 5.56 ± 0.37 | 5.31 ± 0.31 | 5.14 ± 0.33 | 5.50 ± 0.33 | 5.50 ± 0.33 | 5.14 ± 0.33 |
| [1]Number of estrous cycles | 2.78 ± 0.15 | 2.88 ± 0.12 | 3.00 ± 0.17 | 2.80 ± 0.13 | 2.80 ± 0.13 | 3.00 ± 0.17 |
| [2]Proestrus (%) | 26.67 (23.33 – 30.00) | 20.00 (20.00 – 31.67) | 26.67 (23.33 – 36.67) | 30.00 (26.67 – 33.33) | 26.67 (20.00 – 33.33) | 26.67 (20.00 – 33.33) |
| [2]Estrus (%) | 26.67 (20.00 – 26.67) | 26.67 (26.67 – 33.33) | 26.67 (20.00 – 26.67) | 26.67 (20.00 – 26.67) | 20.00 (20.00 – 28.33) | 26.67 (20.00 – 26.67) |
| [2]Metaestrus (%) | 26.67 (16.67 – 33.33) | 26.67 (15.00 – 31.67) | 13.33 (13.33 – 33.33) | 23.33 (18.33 – 26.67) | 30.00 (20.00 – 33.33) | 26.67 (20.00 – 26.67) |
| [2]Diestrus (%) | 20.00 (13.33 – 36.67) | 23.33 (15.00 – 36.67) | 26.67 (16.67 – 33.33) | 23.33 (18.33 – 28.33) | 23.33 (13.33 – 30.00) | 26.67 (16.67 – 26.67) |

Values expressed as mean ± standard error of mean (SEM)[1] or median and interquartile intervals[2], $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 2.** Reproductive performance and fertility test in the female offspring from experimental groups after sexual behavior testing on PND 75.

| | Experimental Groups (n=9 or 10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Gestational rate (%) | 75% | 100% | 88.88% | 100% | 90% | 80% |
| [1]Fertility test (%) | 100 (90.80 – 100) | 96.67 (27.23 – 100) | 93.54 (88.08 – 98.61) | 93.30 (87.98 – 100) | 100 (75.15 – 100) | 93.33 (88.24 – 100) |
| [1]Post-implantation loss (%) | 3.12 (0 – 8.60) | 0.0 (0 – 7.14) | 5.88 ( 0 – 8.01) | 7.69 (0 – 13.33) | 11.76 (0 – 14.84) | 0 (0 – 10.00) |
| [1]Sex ratio (M:F) | 1.00 (0.67 – 1.87) | 1.40 (0.40 – 2.83) | 1.54 (1.19 – 2.00) | 1.43 (0.66 – 2.00) | 1.17 (0.40 – 1.50) | 1.33 (1.00 – 1.75) |
| [2]Body weight (GD20) | 374.70 ± 13.39 | 353.70 ± 14.70 | 359.30 ± 12.28 | 348.10 ± 9.87 | 340.00 ± 16.91 | 340.30 ± 20.01 |
| [2]Gravid uterus | 64.40 ± 3.58 | 57.15 ± 8.67 | 62.64 ± 4.82 | 61.94 ± 1.24 | 50.13 ± 9.51 | 56.41 ± 7.46 |
| [2]Male fetus weight | 2.87 ± 0.10 | 2.83 ± 0.16 | 2.96 ± 0.06 | 3.05 ± 0.08 | 3.21 ± 0.07 | 2.97 ± 0.17 |
| [2]Female fetus weight | 2.56 ± 0.14 | 2.69 ± 0.13 | 2.85 ± 0.11 | 2.89 ± 0.06 | 3.01 ± 0.05 | 2.87 ± 0.15 |
| [2]Placenta weight from male fetus | 0.57 ± 0.02 | 0.53 ± 0.02 | 0.55 ± 0.01 | 0.53 ± 0.01 | 0.53 ± 0.02 | 0.51 ± 0.02 |
| [2]Placenta weight from female fetus | 0.54 ± 0.01 | 0.49 ± 0.02 | 0.52 ± 0.03 | 0.52 ± 0.02 | 0.55 ± 0.04 | 0.49 ± 0.02 |

Values expressed as median and interquartile intervals[1] or mean ± SEM[2], $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 3.** Body weight and reproductive organ weights in the female offspring from experimental groups on first estrus after PND 42.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Body weight (g) | 142.50 ± 4.71 | 142.20 ± 4.57 | 139.70 ± 4.36 | 143.40 ± 3.42 | 141.60 ± 3.14 | 143.00 ± 3.72 |
| Ovaries (mg) | 61.27 ± 4.24 | 65.06 ± 6.65 | 55.93 ± 3.20 | 56.47 ± 4.54 | 60.75 ± 3.83 | 57.78 ± 2.44 |
| Ovaries (mg/100g BW) | 43.26 ± 3.13 | 46.21 ± 5.36 | 40.10 ± 2.02 | 39.32 ± 2.96 | 43.24 ± 3.07 | 40.67 ± 2.24 |
| Oviducts (mg) | 26.02 ± 1.18 | 25.13 ± 1.65 | 23.40 ± 1.08 | 24.82 ± 1.65 | 24.47 ± 1.38 | 23.14 ± 1.55 |
| Oviducts (mg/100g BW) | 18.49 ± 1.16 | 17.76 ± 1.20 | 16.83 ± 0.83 | 17.36 ± 1.15 | 17.35 ± 1.00 | 16.21 ± 1.03 |
| Uterus (mg) | 298.80 ± 20.25 | 287.70 ± 32.93 | 317.10 ± 26.78 | 300.30 ± 41.37 | 281.10 ± 8.82 | 274.10 ± 20.57 |
| Uterus (mg/100g BW) | 212.20 ± 18.76 | 200.80 ± 19.49 | 226.10 ± 15.91 | 212.30 ± 31.31 | 199.50 ± 7.43 | 191.60 ± 13.70 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 4.** Body weight and reproductive organ weights in the female offspring from experimental groups on first estrus after PND 75.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Body weight (g) | 247.30 ± 7.94 [a] | 248.40 ± 7.87 [a] | 245.80 ± 3.65 [a] | 231.60 ± 4.89 [a] | 241.30 ± 4.29 [a] | 239.00 ± 6.85 [a] |
| Ovaries (mg) | 87.70 ± 4.17 [a] | 86.98 ± 5.03 [a] | 88.61 ± 4.96 [a] | 85.50 ± 5.16 [a] | 88.95 ± 6.38 [a] | 107.80 ± 6.06 [b] |
| Ovaries (mg/100g BW) | 35.54 ± 1.49 [a] | 35.06 ± 1.84 [a] | 36.23 ± 2.34 [a] | 36.94 ± 2.16 [a] | 37.08 ± 2.61 [a] | 45.31 ± 2.61 [b] |
| Oviducts (mg) | 28.76 ± 0.88 [a] | 28.56 ± 1.41 [a] | 27.17 ± 1.25 [a] | 28.75 ± 1.14 [a] | 30.06 ± 1.71 [a] | 33.19 ± 2.12 [a] |
| Oviducts (mg/100g BW) | 11.67 ± 0.32 [a] | 11.60 ± 0.76 [a] | 11.06 ± 0.50 [a] | 12.43 ± 0.46 [a] | 12.61 ± 0.89 [a] | 13.97 ± 0.99 [a] |
| Uterus (mg) | 355.00 ± 17.92 [a] | 343.40 ± 19.23 [a] | 369.90 ± 12.98 [a] | 347.10 ± 12.82 [a] | 366.80 ± 17.40 [a] | 398.10 ± 33.05 [a] |
| Uterus (mg/100g BW) | 143.80 ± 6.05 [a] | 139.10 ± 8.75 [a] | 151.00 ± 6.23 [a] | 150.50 ± 6.48 [a] | 153.10 ± 8.62 [a] | 167.00 ± 13.05 [a] |

Values expressed as mean ± SEM, $p < 0.05$. Two-way ANOVA followed by Bonferroni's test.

**Table 5.** Vital organ weights in the female offspring from experimental groups on first estrus after PND 42.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Pituitary (mg) | 6.23 ± 0.33 | 7.24 ± 0.68 | 7.07± 0.37 | 7.10 ± 0.57 | 6.69 ± 0.41 | 6.11 ± 0.40 |
| Pituitary (mg/100g BW) | 4.39 ± 0.24 | 5.07 ± 0.41 | 5.06 ± 0.20 | 4.93 ± 0.36 | 4.73 ± 0.28 | 4.29 ± 0.30 |
| Thyroid (mg) | 10.62 ± 0.59 | 10.81 ± 0.62 | 11.40 ± 0.73 | 11.14 ± 0.99 | 10.80 ± 0.70 | 11.15 ± 0.70 |
| Thyroid (mg/100g BW) | 7.53 ± 0.50 | 7.59 ± 0.34 | 8.18 ± 0.51 | 7.79 ± 0.68 | 7.72 ± 0.60 | 7.81 ± 0.48 |
| Liver (g) | 12.03 ± 0.57 | 12.25 ± 0.81 | 11.58 ± 0.52 | 10.71 ± 0.39 | 12.16 ± 0.47 | 11.97 ± 0.42 |
| Liver (g/100g BW) | 7.79 ± 0.31 | 7.82 ± 0.33 | 7.20 ± 0.25 | 7.52 ± 0.20 | 7.49 ± 0.17 | 7.33 ± 0.29 |
| Adrenal (mg) | 25.26± 0.70 | 24.90 ± 1.26 | 23.97 ± 1.53 | 22.48 ± 1.74 | 23.39 ± 1.74 | 24.38 ± 1.51 |
| Adrenal (mg/100g BW) | 17.80 ± 0.52 | 17.70 ± 1.16 | 17.17 ± 1.00 | 15.78 ± 1.29 | 16.49 ± 1.23 | 17.19 ± 1.28 |
| Kidney (g) | 760.50 ± 24.67 | 722.70 ± 30.32 | 731.70 ± 20.37 | 734.40 ± 15.85 | 730.60 ± 13.52 | 746.20 ± 25.64 |
| Kidney (g/100g BW) | 535.10 ± 14.87 | 508.20 ± 14.06 | 525.90 ± 15.05 | 513.80 ± 12.08 | 517.90 ± 12.50 | 522.20 ± 14.49 |
| Brain (g) | 1.63 ± 0.05 | 1.66 ± 0.02 | 1.66 ± 0.03 | 1.70 ± 0.02 | 1.71 ± 0.03 | 1.67 ± 0.02 |
| Brain (g/100g BW) | 1.15 ± 0.04 | 1.17 ± 0.03 | 1.19 ± 0.04 | 1.19 ± 0.03 | 1.21 ± 0.03 | 1.17 ± 0.02 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Table 6.** Vital organ weights in the female offspring from experimental groups on first estrus after PND 75.

| | Experimental Groups (n=10) | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **3 mg** | **10 mg** | **AA** | **3 mg + AA** | **10 mg + AA** |
| Pituitary (mg) | 12.63 ± 0.71 | 12.39 ± 0.56 | 11.50 ± 0.58 | 10.57 ± 1.21 | 11.80 ± 0.52 | 10.92 ± 0.86 |
| Pituitary (mg/100g BW) | 5.11 ± 0.26 | 4.99 ± 0.20 | 4.68 ± 0.24 | 4.60 ± 0.52 | 4.89 ± 0.17 | 4.60 ± 0.36 |
| Thyroid (mg) | 14.49 ± 1.31 | 16.11 ± 0.90 | 13.69 ± 0.69 | 15.38 ± 0.79 | 13.19 ± 0.53 | 15.28 ± 0.68 |
| Thyroid (mg/100g BW) | 7.53 ± 0.50 | 6.98 ± 0.69 | 5.60 ± 0.32 | 6.69 ± 0.43 | 5.51 ± 0.28 | 6.42 ± 0.30 |
| Liver (g) | 8.83 ± 0.37 | 8.85 ± 0.38 | 8.74 ± 0.21 | 8.54 ± 0.25 | 8.48 ± 0.25 | 8.76 ± 0.29 |
| Liver (g/100g BW) | 3.57 ± 0.06 | 3.56 ± 0.08 | 3.56 ± 0.09 | 3.69 ± 0.05 | 3.52 ± 0.05 | 3.67 ± 0.09 |
| Adrenal (mg) | 51.69 ± 2.85 | 48.15 ± 4.57 | 47.62 ± 2.20 | 48.13 ± 2.19 | 47.08 ± 1.62 | 45.19 ± 2.59 |
| Adrenal (mg/100g BW) | 21.04 ± 1.30 | 19.23 ± 1.46 | 19.35 ± 0.77 | 20.85 ± 1.04 | 19.64 ± 0.79 | 18.97 ± 1.07 |
| Kidney (g) | 1.01 ± 0.04 | 0.93 ± 0.03 | 0.93 ± 0.03 | 0.91 ± 0.93 | 0.91 ± 0.03 | 0.92 ± 0.03 |
| Kidney (g/100g BW) | 408.00 ± 14.58 | 374.60 ± 9.69 | 380.10 ± 10.96 | 393.00 ± 13.01 | 378.40 ± 8.74 | 388.00 ± 11.21 |
| Brain (g) | 1.86 ± 0.02 | 1.80 ± 0.02 | 1.79 ± 0.02 | 1.78 ± 0.03 | 1.86 ± 0.03 | 1.85 ± 0.03 |
| Brain (g/100g BW) | 0.75 ± 0.02 | 0.73 ± 0.02 | 0.73 ± 0.01 | 0.77 ± 0.01 | 0.77 ± 0.02 | 0.78 ± 0.02 |

Values expressed as mean ± SEM, $p > 0.05$. Two-way ANOVA followed by Bonferroni's test. BW = Body weight.

**Figure 1.**

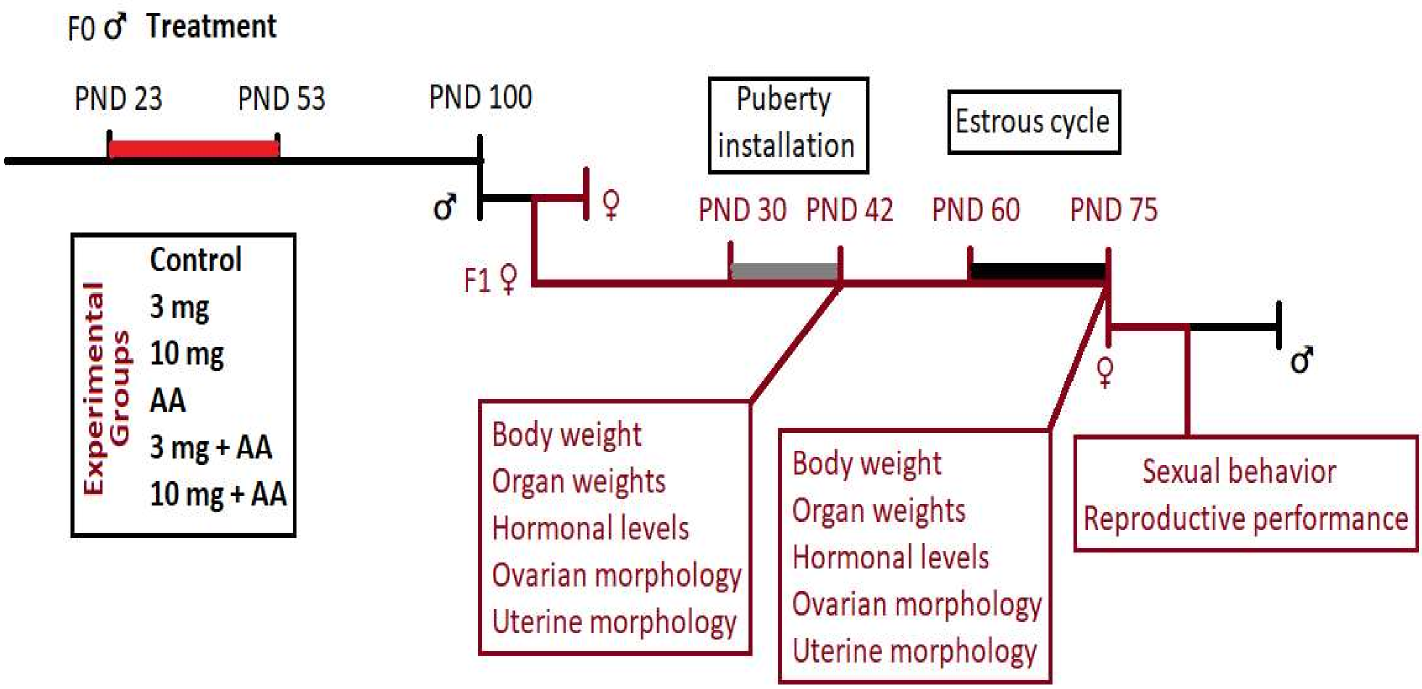

F0 ♂ Treatment
PND 23
PND 53
PND 100
Experimental Groups
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA
♂
♀
F1 ♀
Puberty installation
Estrous cycle
PND 30
PND 42
PND 60
PND 75
Body weight
Organ weights
Hormonal levels
Ovarian morphology
Uterine morphology
Body weight
Organ weights
Hormonal levels
Ovarian morphology
Uterine morphology
♀
♂
Sexual behavior
Reproductive performance

**Figure 2.**

| Parameter | Differences between groups | |
|---|---|---|
| | 42PND | 75PND |
| Vaginal opening | = | = |
| First estrus | = | = |
| Estrous cycle | - | = |
| FSH | = | = |
| LH | **3 mg+AA ↑**<br>**10 mg+AA ↑** | = |
| Progesterone | = | = |
| Primordial/primary follicles | = | = |
| Preantral follicles | = | = |
| Antral follicles | = | = |
| Atretic follicles | = | = |
| Corpora lutea | **10 mg ↓**<br>**10 mg+AA ↓** | = |
| Corpora lutea area | - | **10 mg+AA↑** |
| Uterine area | = | = |
| Perimetrium | = | = |
| Miometrium | = | = |
| Endometrial stroma | = | = |
| Luminal epithelium | = | **3 mg ↓** |
| Luminal area | = | = |
| Number of endometrial glands | = | = |
| Lordosis | - | = |
| Reproductive performance | - | = |
| Body weight | - | = |
| Reproductive organ weights | = | **Ovary**<br>**10 mg+AA ↑** |
| Vital organ weights | = | = |

**Figure 3.**

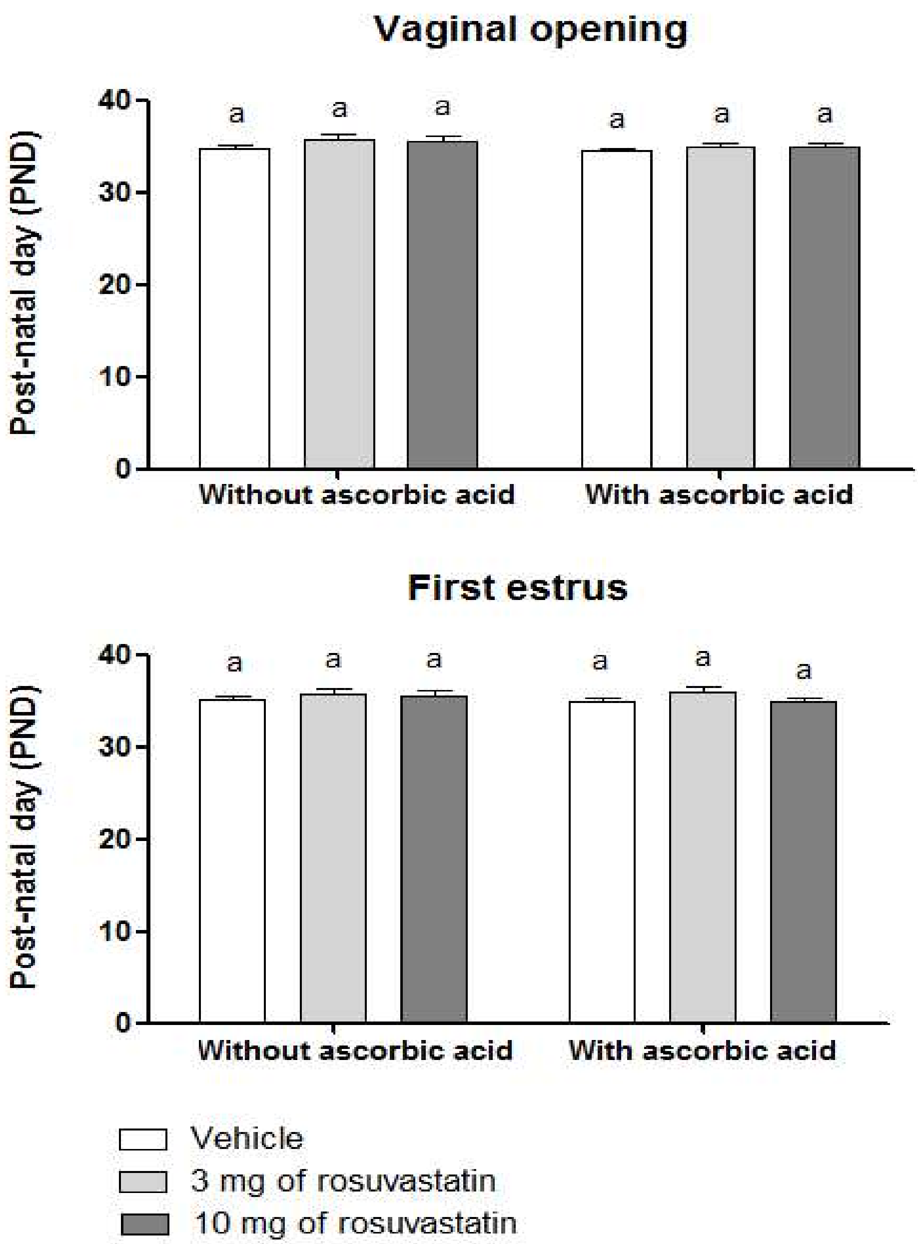

Vaginal opening
Post-natal day (PND)
40
30
20
10
0
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
First estrus
Post-natal day (PND)
40
30
20
10
0
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 4.**

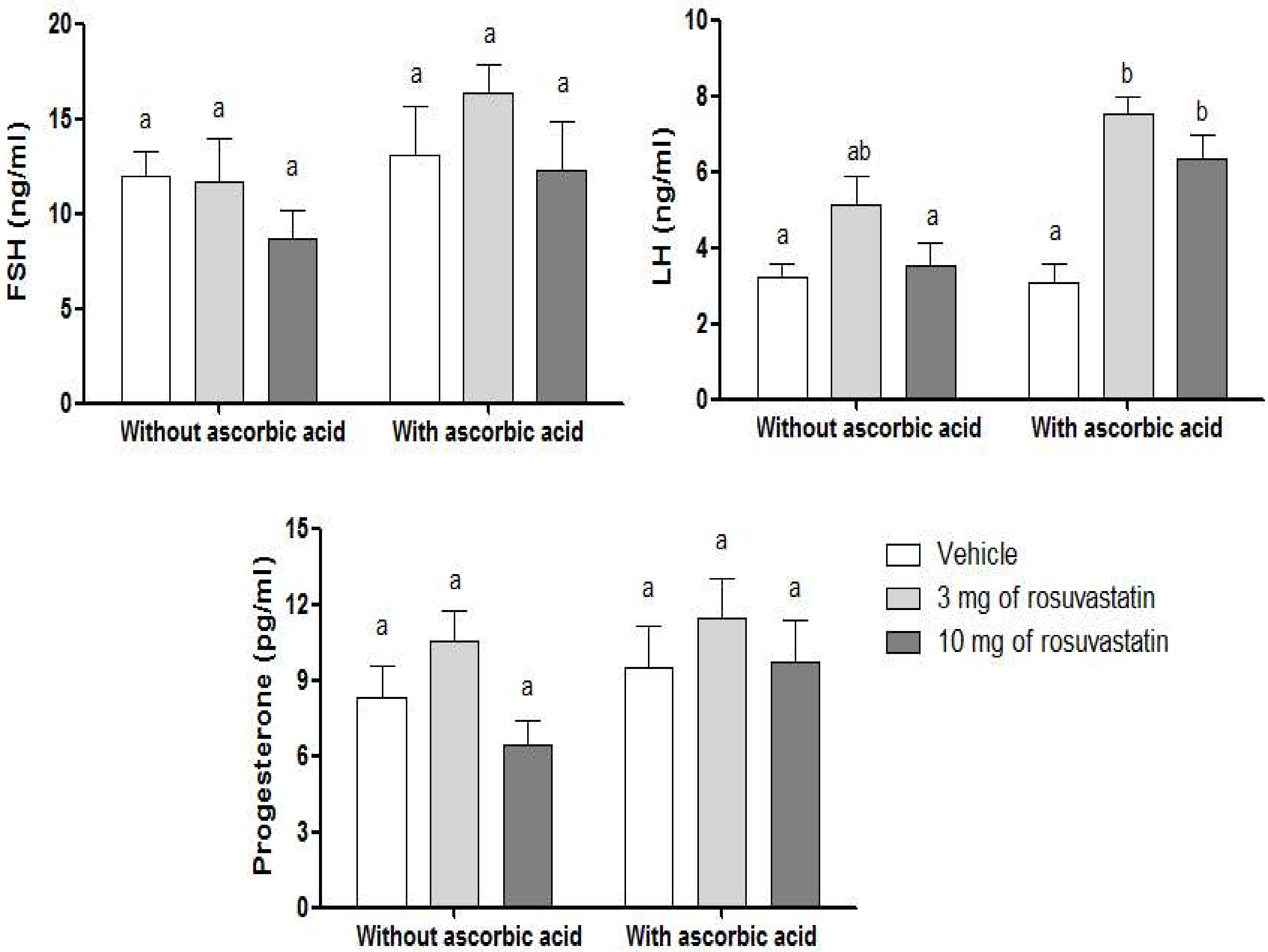

FSH (ng/ml)
Without ascorbic acid
With ascorbic acid
LH (ng/ml)
Progesterone (pg/ml)
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 5.**

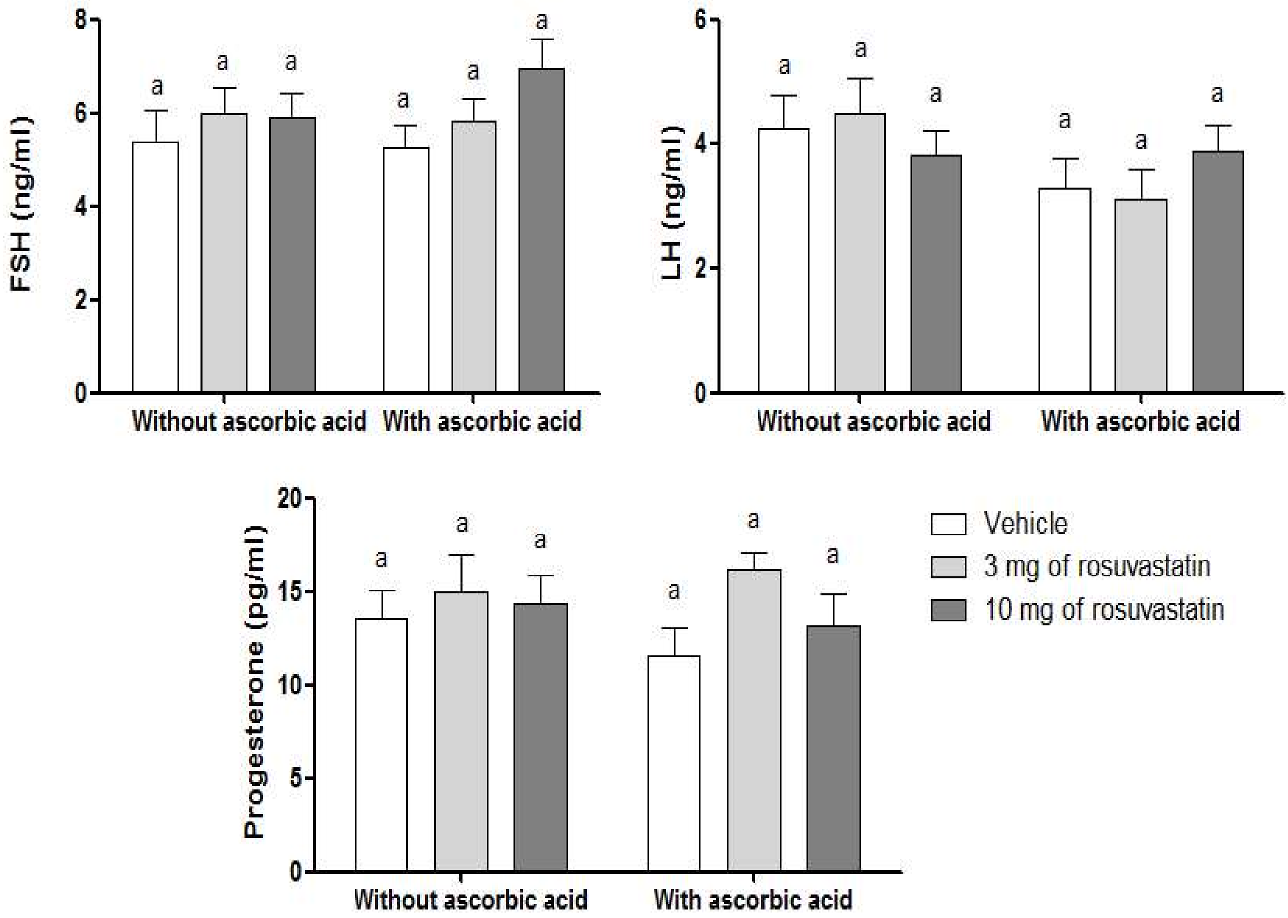

FSH (ng/ml)
8
6
4
2
0
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
LH (ng/ml)
6
4
2
0
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Progesterone (pg/ml)
20
15
10
5
0
a
a
a
a
a
a
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 6.**

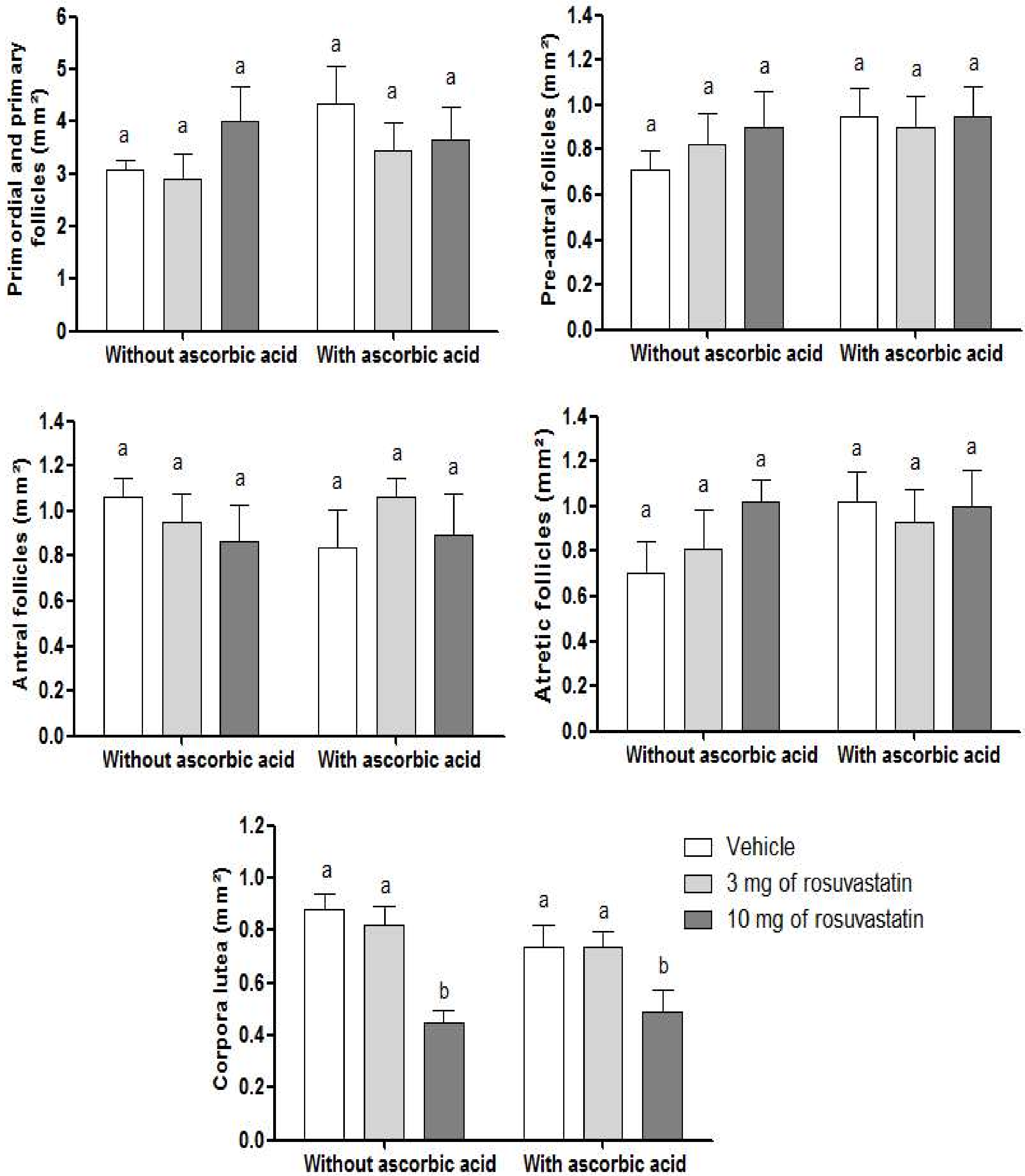

Primordial and primary follicles (mm²)
Without ascorbic acid
With ascorbic acid
Pre-antral follicles (mm²)
Antral follicles (mm²)
Atretic follicles (mm²)
Corpora lutea (mm²)
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 7.**

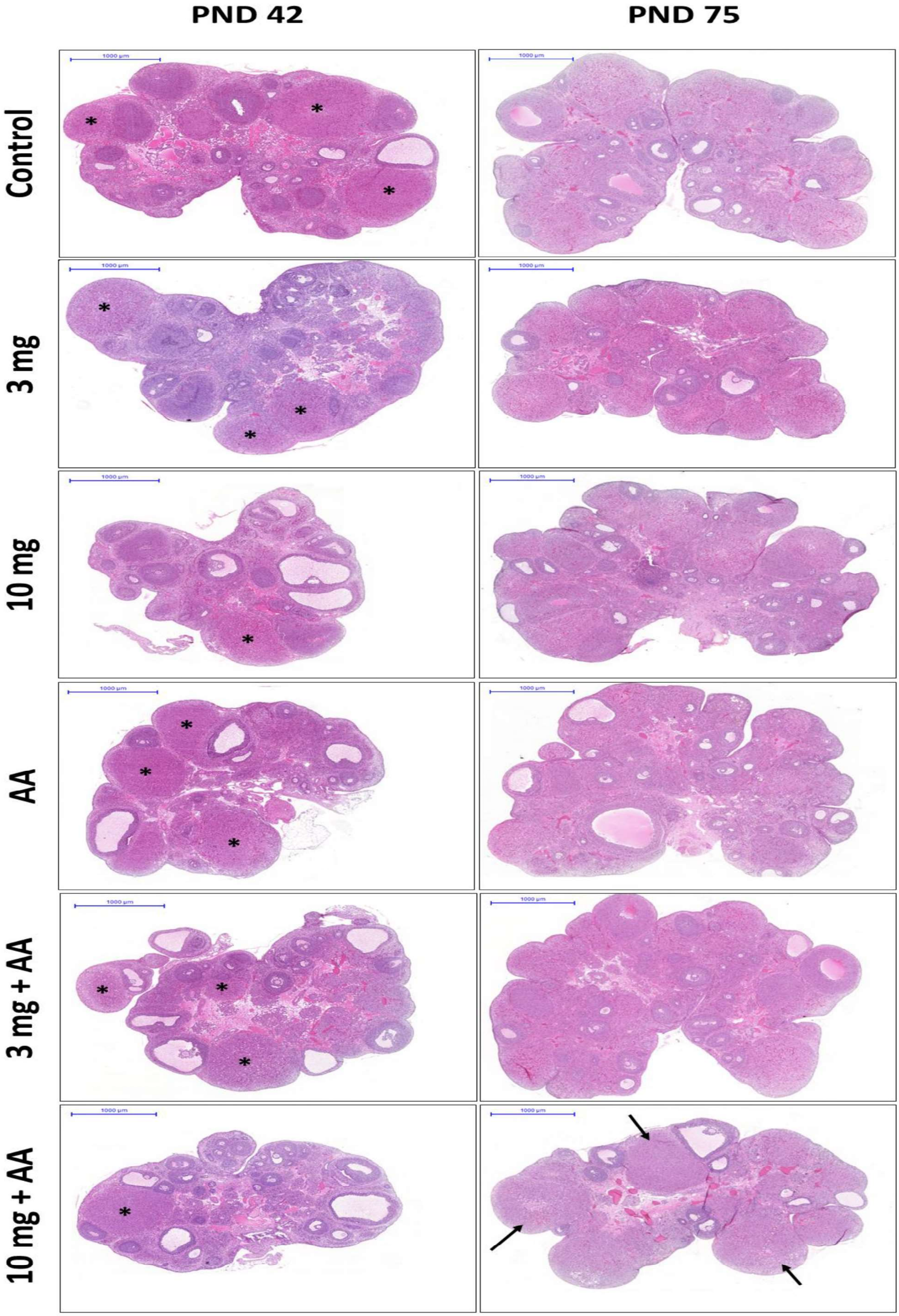


**Figure 8.**

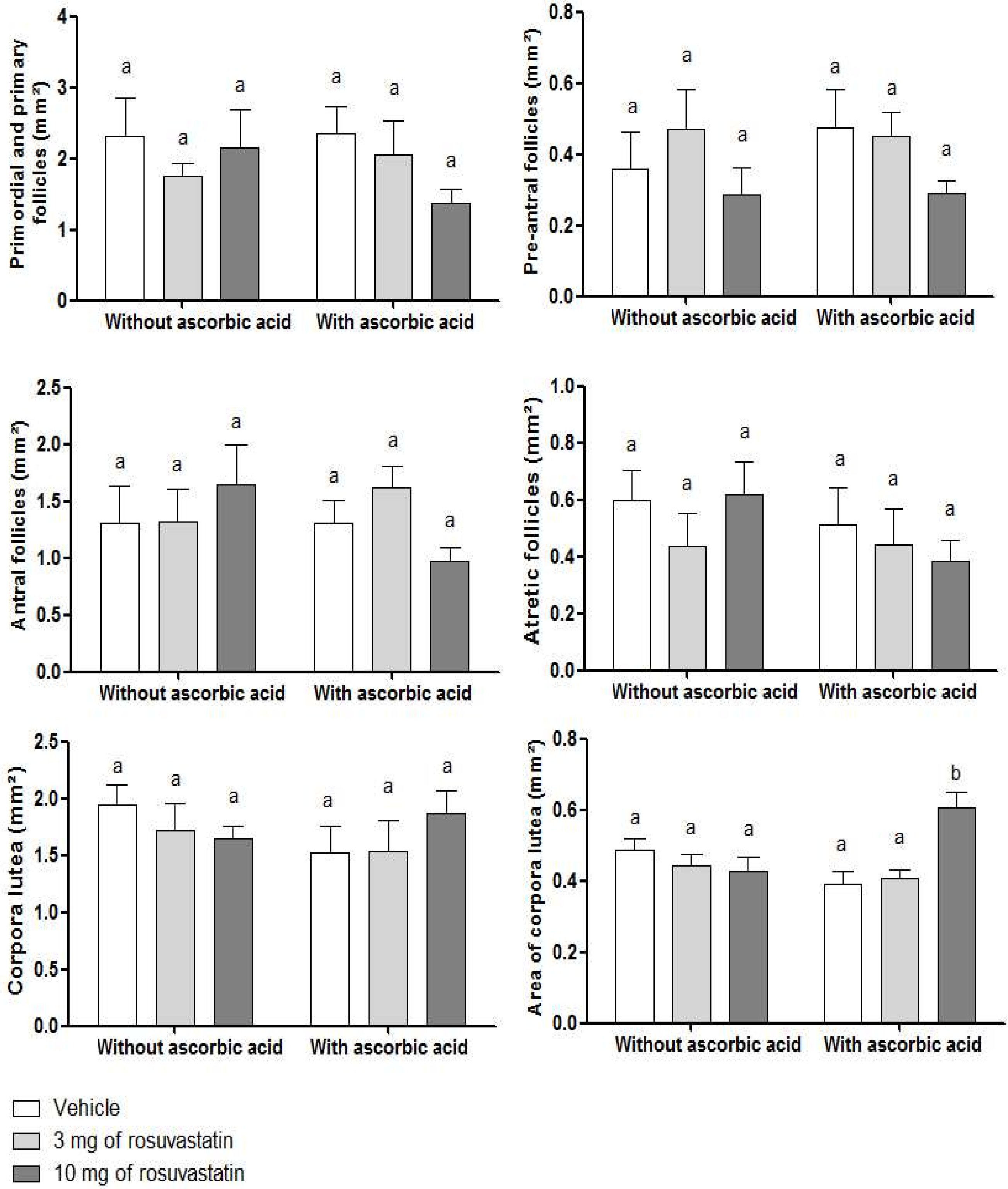

Primordial and primary follicles (mm²)
Pre-antral follicles (mm²)
Antral follicles (mm²)
Atretic follicles (mm²)
Corpora lutea (mm²)
Area of corpora lutea (mm²)
Without ascorbic acid
With ascorbic acid
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 9.**

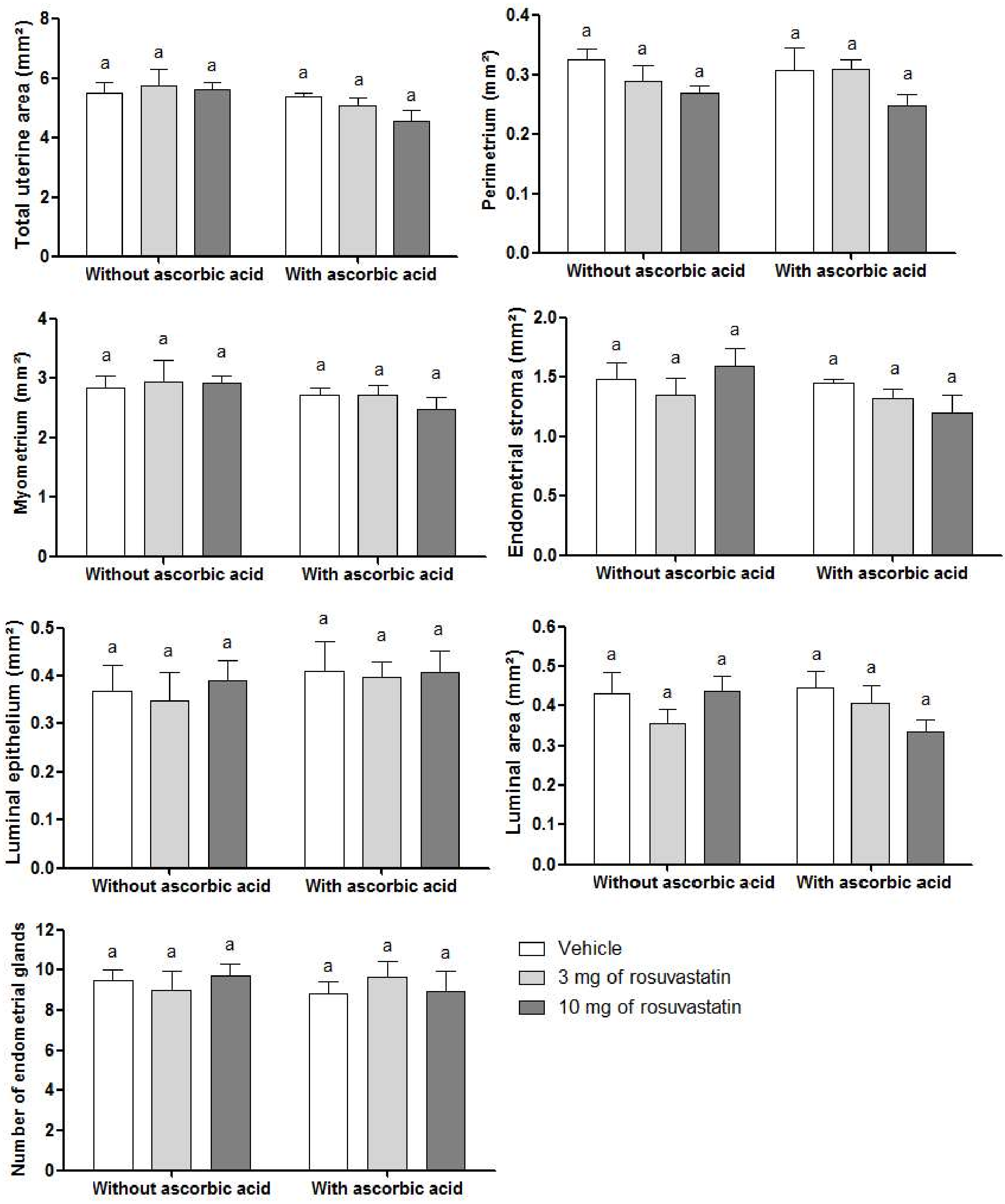

Total uterine area (mm²)
Perimetrium (mm²)
Myometrium (mm²)
Endometrial stroma (mm²)
Luminal epithelium (mm²)
Luminal area (mm²)
Number of endometrial glands
Without ascorbic acid
With ascorbic acid
a
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 10.**

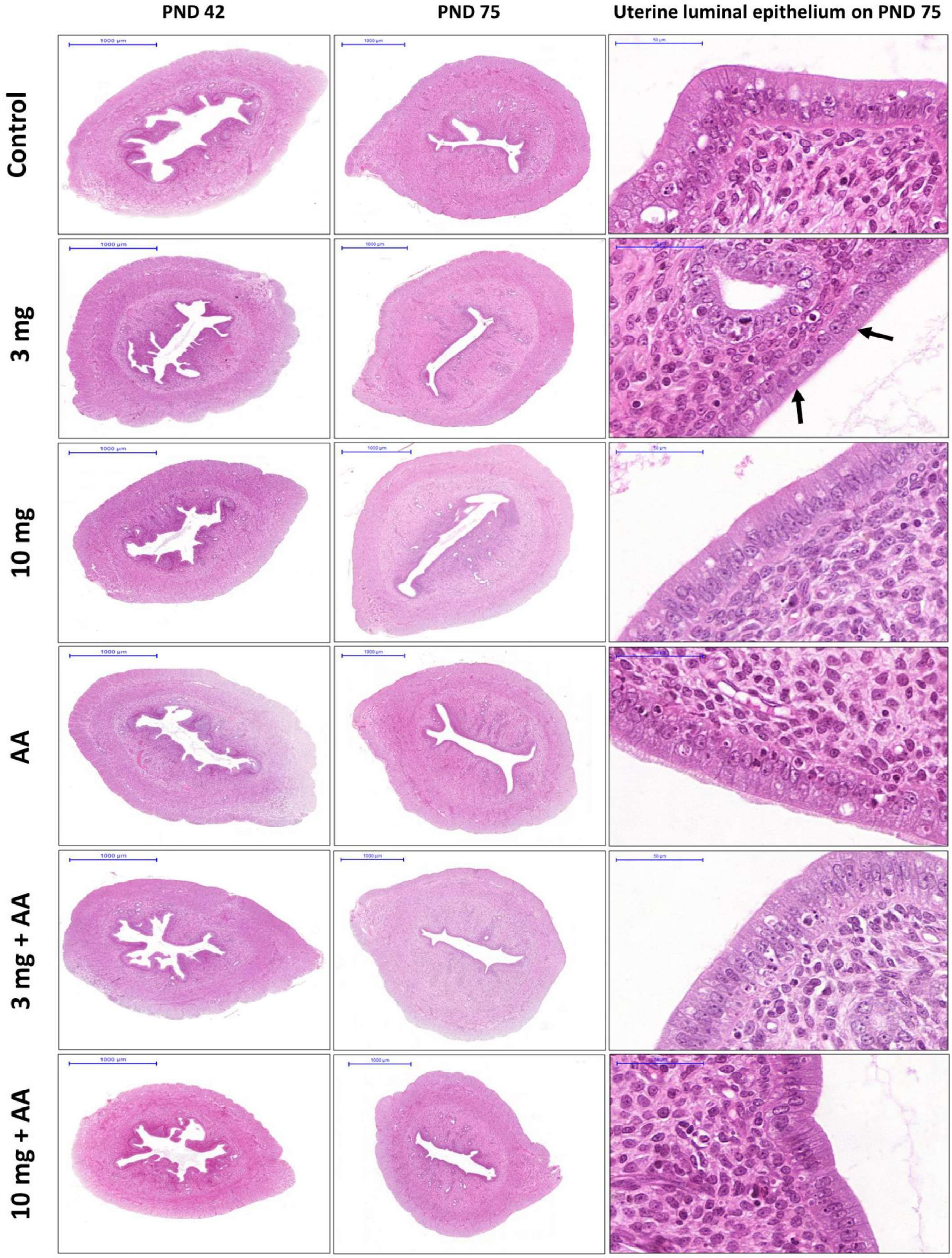

PND 42
PND 75
Uterine luminal epithelium on PND 75
Control
3 mg
10 mg
AA
3 mg + AA
10 mg + AA

**Figure 11.**

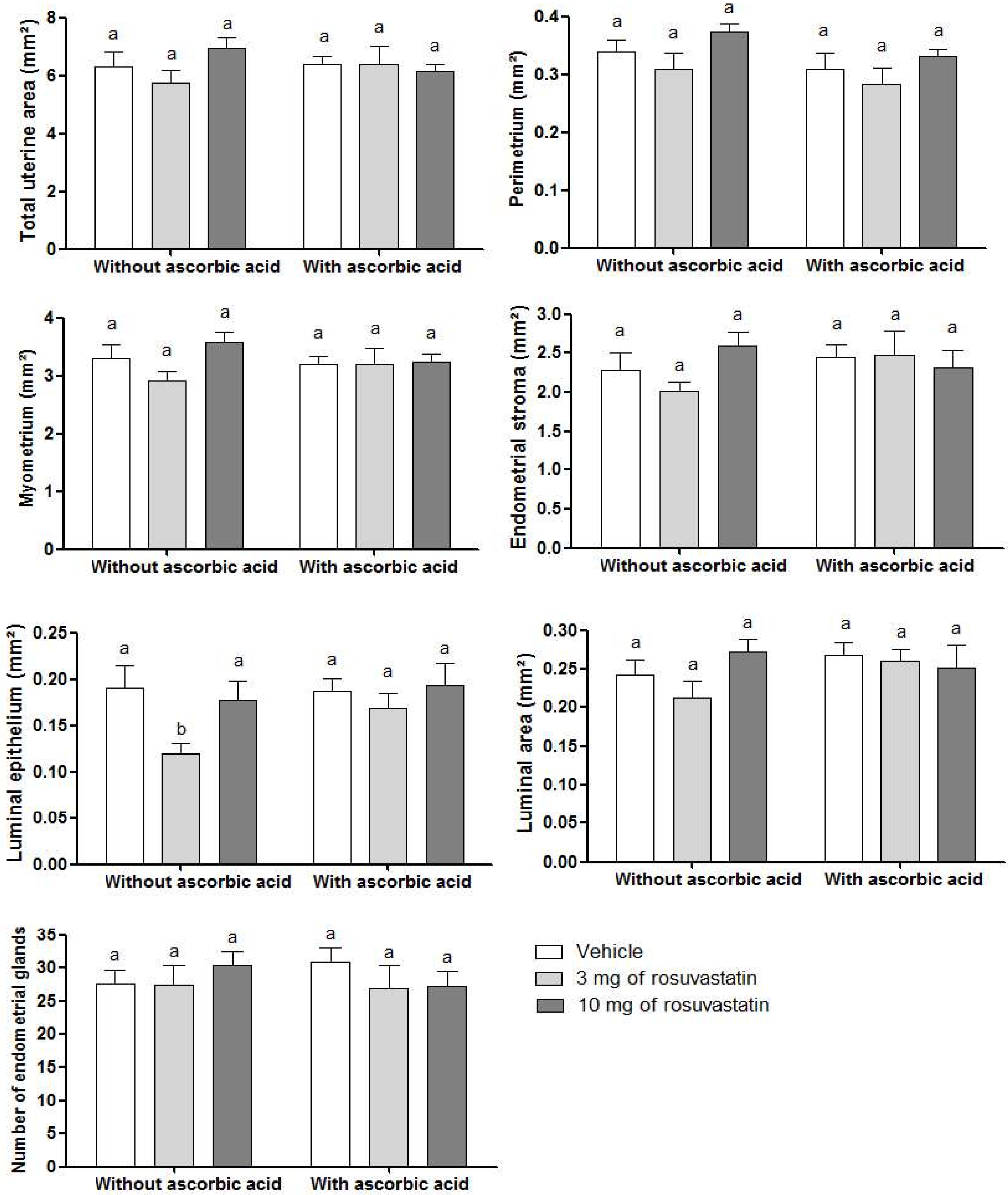
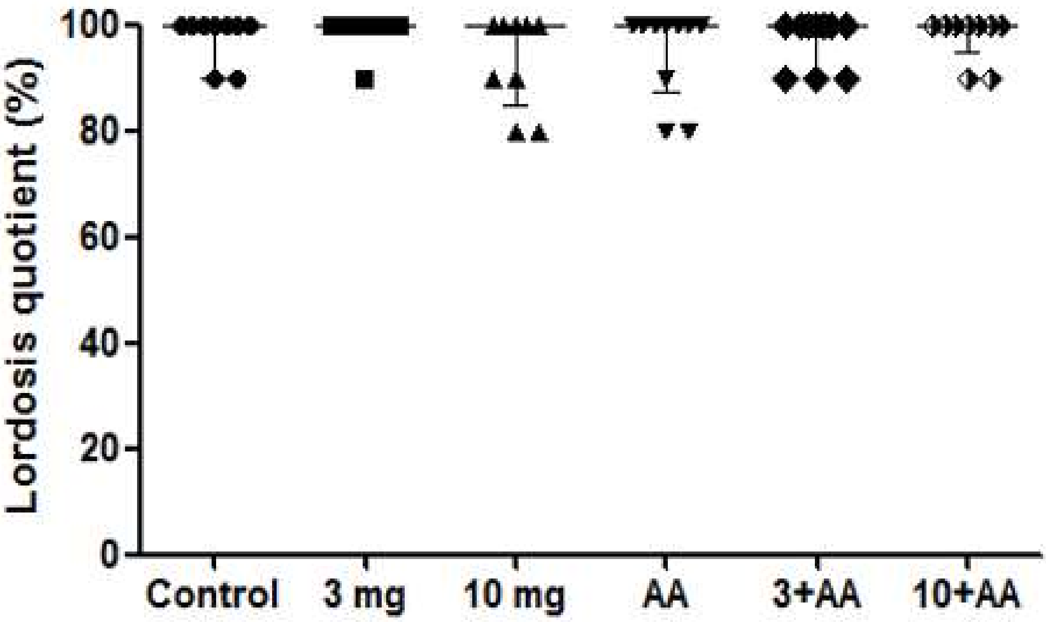

Total uterine area (mm²)
Perimetrium (mm²)
Myometrium (mm²)
Endometrial stroma (mm²)
Luminal epithelium (mm²)
Luminal area (mm²)
Number of endometrial glands
Without ascorbic acid
With ascorbic acid
a
b
Vehicle
3 mg of rosuvastatin
10 mg of rosuvastatin

**Figure 12.**


Lordosis quotient (%)
100
80
60
40
20
0
Control
3 mg
10 mg
AA
3+AA
10+AA

# Resultados Gerais

## RESULTADOS GERAIS

A exposição de ratos machos juvenis à rosuvastatina promoveu atraso na instalação da puberdade, diminuiu a produção espermática diária e atrasou a maturação do epitélio seminífero e a diferenciação epididimária no período pós-natal. Além disso, houve depleção androgênica, redução na marcação nuclear de receptores androgênicos nas células de Sertoli nos estágios IX-XIII e aumento na taxa de morte de células germinativas nos grupos tratados com a estatina, em relação ao grupo controle. O grupo exposto à menor dose da estatina demonstrou aumento na atividade da enzima catalase testicular, provavelmente devido ao aumento nos níveis de peróxido de hidrogênio. A suplementação com ascórbico foi capaz de diminuir os danos reprodutivos promovidos pela rosuvastatina e aumentar a capacidade antioxidante ao final do tratamento hipolipemiante, no DPN 53.

Na maturidade sexual, os animais que foram expostos à rosuvastatina na pré-puberdade apresentaram persistência das seguintes alterações: concentrações diminuídas de testosterona, diminuição da marcação nuclear para receptores androgênicos nas células de Sertoli nos estágios de IX-XIII e aumento da frequência da morte celular. Ademais, houve diminuição da qualidade espermática, alterações ultraestruturais no espermatozoide, aumento na fragmentação do DNA espermático, maior peroxidação lipídica e atividade da enzima catalase, e alterações histopatológicas no epidídimo dos animais que foram tratados com a estatina, em ambas as doses.

No DPN 110, o grupo exposto à maior dose da rosuvastatina apresentou redução na marcação citoplasmática de receptores de estrógeno alfa nas espermátides redondas e alongadas e nas células de Leydig, bem como redução de marcação nuclear nas células de Leydig. Além disso, houve ainda aumento na perda pós-implantação após acasalamento natural dos animais expostos a dose de 10 mg da estatina com fêmeas não tratadas. O ácido ascórbico foi capaz de prevenir parcialmente os danos reprodutivos gerados pela exposição pré-puberal à rosuvastatina.

A prole masculina dos animais expostos à estatina mostrou depleção androgênica, aumento na frequência de morte de células germinativas e no dano do DNA espermático, redução na motilidade e produção espermática, e hiperplasia de células claras na cauda proximal do epidídimo. A co-administração de ácido ascórbico aos pais foi capaz de prevenir, pelo menos parcialmente, os danos reprodutivos na prole masculina promovidos pela exposição paterna à estatina.

A prole feminina dos animais expostos à maior dose de rosuvastatina associada ou não ao ácido ascórbico exibiu atraso no desenvolvimento ovariano, mas sem afetar a morfologia e função ovariana na maturidade sexual. As fêmeas cujos pais foram expostos a menor dose da estatina apresentaram ainda menor área e altura do epitélio luminal uterino, o que sugere menores concentrações de estradiol em relação ao grupo controle. O ácido ascórbico, quando co-administrado para os pais na pré-puberdade, foi capaz de restaurar a área e altura do epitélio luminal uterino na prole feminina.

# Conclusões

# CONCLUSÕES

Os resultados obtidos ratificam a hipótese do trabalho, uma vez que as doses de 3 e 10 mg/Kg/dia de rosuvastatina administradas para ratos púberes, promoveram, na geração $F_0$, atraso na instalação da puberdade, depleção androgênica, diminuição da qualidade espermática e alterações na morfologia testicular e epididimária. Além disso, houve aumento no dano do DNA espermático em ambas as doses e aumento da taxa de perda pós-implantação nas fêmeas acasaladas com ratos expostos à maior dose de rosuvastatina. A suplementação com ácido ascórbico mostrou efeitos benéficos, ajudando a reverter parcialmente os danos reprodutivos causados pela administração de rosuvastatina na pré-puberdade, devido aos seus potenciais efeitos antioxidantes e a capacidade de estimular a esteroidogênese.

A exposição paterna à rosuvastatina foi capaz de promover efeitos reprodutivos adversos na prole masculina e feminina dos animais expostos. A prole masculina apresentou aumento na taxa de morte de células germinativas, depleção androgênica, redução da motilidade e da produção espermática, bem como aumento na fragmentação do DNA. As fêmeas demonstraram atraso na maturação ovariana e menor epitélio luminal uterino nos grupos cujos pais foram expostos às doses de 10 e 3 mg, respectivamente. Deste modo, a exposição paterna à estatina foi capaz de interferir geneticamente, além de possivelmente promover alterações epigenéticas no DNA espermático paterno, afetando assim os parâmetros reprodutivos de sua prole. O ácido ascórbico, quando administrado para os pais, foi capaz de proteger o espermatozoide de alguma maneira, provavelmente devido ao seu papel na desmetilação do DNA, e diminuir o dano reprodutivo na prole dos animais expostos à estatina.

Considerando-se a prevalência de obesidade em crianças e adolescentes, os resultados do presente trabalho apontam para o risco de danos reprodutivos imediatos e tardios decorrentes do uso de rosuvastatina como fármaco hipolipemiante e o papel do ácido ascórbico prevenindo, pelo menos em parte, os danos à saúde reprodutiva provocados pela estatina.

# Referências da Introdução

## REFERÊNCIAS BIBLIOGRÁFICAS

**Adam O and Laufs U** (2008) Antioxidative effects of statins. *Archives of Toxicology* **82** 885–892.

**Aebi H** (1984) Catalase in vitro. *Methods in Enzymology* **105** 121–126.

**Agarwal A, Prabhakaran SA and Said TM** (2005) Prevention of Oxidative Stress Injury to Sperm. *Journal of Andrology* **26** 654–660.

**Allen SC and Mamotte CDS** (2017) Pleiotropic and Adverse Effects of Statins—Do Epigenetics Play a Role? *Journal of Pharmacology and Experimental Therapeutics* **362** 319–326.

**Andersson H, Rehm S, Stanislaus D and Wood CE** (2013) Scientific and Regulatory Policy Committee ( SRPC ) Paper : Assessment of Circulating Hormones in Nonclinical Toxicity Studies. *Toxicology Pathology* **41** 921–934.

**Andrews WW and Ojeda SR** (1981) Secretory profile in conscious, free-moving female rats during the time of puberty. *Endocrinology* **109** 2032–2039.

**Arrigoni E, Re M Del, Fidilio L, Fogli S, Danesi R and Paolo A Di** (2017) Pharmacogenetic foundations of therapeutic efficacy and adverse events of statins. *International Journal of Molecular Sciences* **18** 1–25.

**Arrotéia KF, Garcia P V., Barbieri MF, Justino ML and Pereira LA V.** (2012) The epididymis: Embriology, structure, function and its role in fertilization and infertility. In *Embriology - Updates and Highlights on Classic Topics*, pp 41–66. Ed LA V Pereira. Croatia: InTech.

**Avloniti A, Chatzinikolaou A, Deli C, Vlachopoulos D, Gracia-Marco L, Leontsini D, Draganidis D, Jamurtas A, Mastorakos G and Fatouros I** (2017) Exercise-induced oxidative stress responses in the pediatric population. *Antioxidants* **6** 6.

**Baud O** (2004) Glutathione peroxidase-catalase cooperativity Is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *The Journal of Neuroscience* **24** 1531–1540.

**Bianco SDC** (2012) A potential mechanism for the sexual dimorphism in the onset of puberty and incidence of idiopathic central precocious puberty in children: Sex-specific kisspeptin as an integrator of puberty signals. *Frontiers in Endocrinology* **3** 1–16.

**Blystone CR, Furr J, Lambright CS, Howdeshell KL, Ryan Bryce C, Wilson VS, LeBlanc**

**GA and Gray LE** (2007) Prochloraz inhibits testosterone production at dosages below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague dawley rat. *Toxicological Sciences* **97** 65–74.

**Branham WS and Sheehan DM** (1995) Ovarian and adrenal contributions to postnatal growth and differentiation of the rat uterus. *Biology of Reproduction* **53** 863–872.

**Brody JR and Cunha GR** (1989) Histologic , morphometric , and immunocytochemical analysis of myometrial development in rats and mice : II . Effects of DES on development. *The American Journal of Anatomy* **186** 21–42.

**Bukovsky A, Caudle MR, Svetlikova M, Wimalasena J, Ayala ME and Dominguez R** (2005) Oogenesis in adult mammals, including humans: A review. *Endocrine* **26** 301–316.

**Camarena V and Wang G** (2016) The epigenetic role of vitamin C in health and disease. *Cellular and Molecular Life Sciences* **73** 1645–1658.

**Campo VL and Carvalho I** (2007) Estatinas hipolipêmicas e novas tendências terapêuticas. *Quimica Nova* **30** 425–430.

**Chen H, Ge RS and Zirkin BR** (2009) Leydig cells: From stem cells to aging. *Molecular and Cellular Endocrinology* **306** 9–16.

**Cheng CY and Mruk DD** (2012) The blood-testis barrier and its implications for male contraception. *Pharmacol Rev.* **64** 16–64.

**Choi J-H and Yoo H-W** (2013) Control of puberty: genetics, endocrinology and environment. *Current Opinion in Endocrinology, Diabetes, and Obesity* **20** 62–68.

**Cook S and Kavey REW** (2011) Dyslipidemia and pediatric obesity. *Pediatric Clinics of North America* **58** 1363–1373.

**Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL and Pericak-Vance MA** (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science (New York, N.Y.)* **261** 921–923.

**Corsetti G, Stacchiotti A, Tedesco L, D'Antona G, Pasini E, Dioguardi FS, Nisoli E and Rezzani R** (2011) Essential amino acid supplementation decreases liver damage induced by chronic ethanol consumption in rats. *International Journal of Immunopathology and Pharmacology* **24** 611–619.

**Davis BJ, Travlos G and McShane T** (2001) Reproductive endocrinology and toxicological pathology over the life span of the female rodent. *Toxicologic Pathology* **29** 77–83.

**Drews U** (2000) Local mechanisms in sex specific morphogenesis. *Cytogenetics and Cell Genetics* **91** 72–80.

**Dyche WJ** (1979) A comparative study of the differentiation and involution of the Mullerian duct and Wolffian duct in the male and female fetal mouse. *Journal of Morphology* **162** 175–209.

**Ebling FJP** (2005) The neuroendocrine timing of puberty. *Reproduction* **129** 675–683.

**Edison RJ and Muenke M** (2004) Mechanistic and epidemiologic considerations in the evaluation of adverse birth outcomes following gestational exposure to statins. *American Journal of Medical Genetics. Part A* **131** 287–298.

**Edwards TM, Moore BC and Giellette Jr LJ** (2006) Reproductive dysgenesis in wildlife: a comparative view. *International Journal of Andrology* **29** 109–121.

**Endres M** (2006) Statins: Potential new indications in inflammatory conditions. *Atherosclerosis Supplements* **7** 31–35.

**Eskenazi B, Kidd SA, Marks AR, Sloter E, Block G and Wyrobek AJ** (2005) Antioxidant intake is associated with semen quality in healthy men. *Human Reproduction* **20** 1006–1012.

**Favareto APA, Fernandez CDB, da Silva DAF, Anselmo-Franci JA and Kempinas WDG** (2011a) Persistent Impairment of Testicular Histology and Sperm Motility in Adult Rats Treated with Cisplatin at Peri-Puberty. *Basic and Clinical Pharmacology and Toxicology* **109** 85–96.

**Favareto APA, de Toledo FC and Kempinas WDG** (2011b) Paternal treatment with cisplatin impairs reproduction of adult male offspring in rats. *Reproductive Toxicology* **32** 425–433.

**Fawcett DW, Neaves WB and Flores MN** (1973) Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. *Biology of Reproduction* **9** 500–532.

**Feleszko W, Zagozdzon R, Gołąb J and Jakóbisiak M** (1998) Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice. *European Journal of Cancer* **34** 406–411.

**Fernandes GSA, Gerardin DCC, Assumpção TA, Campos KE, Damasceno DC, Pereira OCM and Kempinas WDG** (2011a) Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats? *Pharmacological Reports* **63**

983–991.

**Fernandes GSA, Fernandez CDB, Campos KE, Damasceno DC, Anselmo-Franci JA and Kempinas WDG** (2011b) Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. *Reproductive Biology and Endocrinology : RB&E* **100** 1–9.

**Fisher MM and Eugster EA** (2014) What is in our environment that effects puberty? *Reproductive Toxicology* **44** 7–14.

**Fonseca FAH** (2005) Pharmacokinetics of statins. *Arquivos Brasileiros de Cardiologia* **85** 9–14.

**França LR, Avelar GF and Almeida FFL** (2005) Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. *Theriogenology* **63** 300–318.

**George FW and Wilson JD** (1994) Gonads and ducts in mammals. In *The Physiology of Reproduction*, 2nd Editio, pp 3–28. Eds E Knobil and JD Neil. New York: Raven Press.

**German Nutrition Society** (2015) New Reference Values for Vitamin C Intake. *Annals of Nutrition & Metabolism* **67** 13–20.

**Giuliano ICB and Caramelli B** (2008) Dislipidemias na infância e na adolescência. *Pediatria (São Paulo)* **29** 275–285.

**Griswold SL and Behringer RR** (2009) Fetal Leydig cell origin and development. *Sexual Development* **3** 1–15.

**Guerra MT, Sanabria M, Cagliarani SV, Leite GAA, Borges C dos S and Kempinas WDG** (2017) Long-Term Effects of in Utero and Lactational Exposure to Butyl Paraben in Female Rats. *Environmental Toxicology* **32** 776–788.

**Habert R and Picon R** (1984) Testosterone, dihydrotestosterone and estradiol-17β levels in maternal and fetal plasma and in fetal testis in the rat. *The Journal of Steroid Biochemistry and Molecular Biology* **21** 193–198.

**Haider SG** (2004) Cell biology of Leydig cells in the testis. *International Review of Cytology* **233** 181–241.

**Heard E and Martienssen RA** (2014) Transgenerational epigenetic inheritance: Myths and mechanisms. *Cell* **157** 95–109.

**Hermo L and Robaire B** (2002) Epididymal cell types and their functions. In *The Epididymis – from Molecules to Clinical Practice*, pp 81–102. Eds B Robaire and BT Hinton. New York: Kluwer Academic/Plenum Publisher.

**Hermo L, Barin K and Robaire B** (1992) Structural differentiation of the epithelial cells of the testicular excurrent duct system of rats during postnatal development. *Anatomical Record* **233** 205–228.

**Heyn R, Makabe S and Motta P** (1998) Ultrastructural dynamics of human testicular cords from 6 to 16 weeks of embryonic development. Study by transmission and high resolution scanning electron microscopy. *Italian Journal of Anatomy and Embryology* **103** 17–29.

**Hillstrom RJ, Yacapin-ammons AK and Lynch SM** (2003) Vitamin C Inhibits Lipid Oxidation in Human HDL. *Biochemical and Molecular Actions of Nutrients* **10** 3047–3051.

**Hirobe S, He WW, Lee MM and Donahoe PK** (1992) Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. *Endocrinology* **131** 854–862.

**Holdgate GA, Ward WHJ and McTaggart F** (2003) Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. *Biochemical Society Transactions* **31** 528–531.

**Hu J, Gray CA and Spencer TE** (2004) Gene expression profiling of neonatal mouse uterine development. *Biology of Reproduction* **70** 1870–1876.

**Huhtaniemi I** (1994) Fetal testis - a very special endocrine organ. *European Journal of Endocrinology* **130** 25–31.

**Hutson JC** (1990) Changes in the concentration and size of testicular macrophages during development. *Biology of Reproduction* **43** 885–890.

**Istvan E** (2003) Statin inhibition of HMG-CoA reductase: A 3-dimensional view. *Atherosclerosis Supplements* **4** 3–8.

**Istvan ES and Deisenhofer J** (2001) Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* **292** 1160–1164.

**Izar MCDO, Fonseca MIH and Fonseca FAH** (2011) Dyslipidemias. *Revista Brasileira de Medicina* **68** 59–73.

**Jiménez EG and Ferre JA** (2011) Estatinas : Características y Efectos sobre el Control Lipídico en el Niño y Adolescente Obeso. *Revista Clínica de Medicina de Familia* **4** 69–75.

**Johnson L., Welsh THJ and Wilker CE** (1997) Anatomy and physiology of the male reproductive system and potential targets of toxicants. In *Comprehensive Toxicology*, pp 5–61. Eds K Boekleheide, R Chapin, C Hoyer and P Harris. New York: Pergamon.

**Josso N, Picard JY, Dacheux JL and Courot M** (1979) Detection of anti-mullerian activity in boar rete testis fluid. *Reproduction* **57** 397–400.

**Jost A, Vigier B, Prépin J and Perchellet JP** (1973) Studies on sex differentiation in mammals. *Recent Progress in Hormone Research* **29** 1–41.

**Klop B, Elte JWF and Cabezas MC** (2013) Dyslipidemia in Obesity: Mechanisms and Potential Targets. *Nutrients* **5** 1218–1240.

**Kwiterovich PO** (2008) Recognition and management of dyslipidemia in children and adolescents. *Journal of Clinical Endocrinology and Metabolism* **93** 4200–4209.

**Lee SK and Kim YS** (2013) Phosphorylation of eIF2α attenuates statin-induced apoptosisby inhibiting the stabilization and translocation of p53 to the mitochondria. *International Journal of Oncology* **42** 810–816.

**Losinno AD, Morales A, Fernandez D and Lopez LA** (2012) Peritubular myoid mells from rat seminiferous tubules contain actin and myosin filaments distributed in two independent layers. *Biology of Reproduction* **86** 150–150.

**Ly L, Chan D, Aarabi M, Landry M, Behan NA, Macfarlane AJ and Trasler J** (2017) Intergenerational impact of paternal lifetime exposures to both folic acid de fi ciency and supplementation on reproductive outcomes and imprinted gene methylation. *Molecular Human Reproduction* **1** 1–17.

**Maekawa M, Kamimura K and Nagano T** (1996) Peritubular myoid cells in the testis: their structure and function. *Archives of Histology and Cytology* **59** 1–13.

**Makker K, Agarwal A and Sharma R** (2009) Oxidative stress & male infertility. *The Indian Journal of Medical Research* **129** 357–367.

**Mantovani A and Fucic A** (2014) Puberty dysregulation and increased risk of disease in adult life: Possible modes of action. *Reproductive Toxicology* **44** 15–22.

**Martin PD, Mitchell PD and Schneck DW** (2002) Pharmacodynamic effects and pharmacokinetics of a new HMG-CoA reductase inhibitor, rosuvastatin, after morning or evening administration in healthy volunteers. *British Journal of Clinical Pharmacology* **54** 472–477.

**Marty MS, Chapin RE, Parks LG and Thorsrud BA** (2003) Development and Maturation of the Male Reproductive System. *Birth Defects Research Part B - Developmental and Reproductive Toxicology* **68** 125–136.

**McGill HC, McMahan CA, Herderick EE, Zieske AW, Malcom GT, Tracy RE and Strong**

**JP** (2002) Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation* **105** 2712–2718.

**McTaggart F** (2003) Comparative pharmacology of rosuvastatin. *Atherosclerosis Supplements* **4** 9–14.

**Mouthuy P-A, Snelling SJB, Dakin SG, Milković L, Gašparović AC, Carr AJ and Žarković N** (2016) Biocompatibility of implantable materials: An oxidative stress viewpoint. *Biomaterials* **109** 55–68.

**Mukhopadhyay PK, Dey A, Mukherjee S and Pradhan NK** (2013) The effect of coadministration of alpha-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. *Journal of Basic and Clinical Physiology and Pharmacology* **24** 245–253.

**Nistal M, Paniagua R, Regadera J, Santamaria L and Amat P** (1986) A quantitative morphological study of human Leydig cells from birth to adulthood. *Cell and Tissue Research* **246** 229–236.

**Okon UA and Utuk II** (2016) Ascorbic acid treatment elevates follicle stimulating hormone and testosterone plasma levels and enhances sperm quality in albino Wistar rats. *Nigerian Medical Journal* **57** 31–36.

**Paltoglou G, Fatouros IG, Valsamakis G, Schoina M, Avloniti A, Chatzinikolaou A, Kambas A, Draganidis D, Mantzou A, Papagianni M *et al.*** (2015) Antioxidation improves in puberty in normal weight and obese boys, in positive association with exercise-stimulated growth hormone secretion. *Pediatric Research* **78** 158–164.

**Pandir D, Kara O and Kara M** (2014) Protective effect of bilberry (Vaccinium myrtillus L.) on cisplatin induced ovarian damage in rat. *Cytotechnology* **66** 677–685.

**Penning-Van Beest FJA, Termorshuizen F, Goettsch WG, Klungel OH, Kastelein JJP and Herings RMC** (2007) Adherence to evidence-based statin guidelines reduces the risk of hospitalizations for acute myocardial infarction by 40%: A cohort study. *European Heart Journal* **28** 154–159.

**Perobelli JE, Alves TR, de Toledo FC, Fernandez CDB, Anselmo-Franci JA, Klinefelter GR and Kempinas WDG** (2012) Impairment on sperm quality and fertility of adult rats after antiandrogen exposure during prepuberty. *Reproductive Toxicology* **33** 308–315.

**Perobelli JE, Patrão MTCC, Fernandez CDB, Sanabria M, Klinefelter GR, Avellar MCW**

**and Kempinas WDG** (2013) Androgen deprivation from pre-puberty to peripuberty interferes in proteins expression in pubertal and adult rat epididymis. *Reproductive Toxicology* **38** 65–71.

**Picut CA, Remick AK, Asakawa MG, Simons ML and Parker GA** (2014a) Histologic Features of Prepubertal and Pubertal Reproductive Development in Female Sprague-Dawley Rats. *Toxicologic Pathology* **42** 403–413.

**Picut CA, Dixon D, Simons ML, Stump DG, Parker GA and Remick AK** (2014b) Postnatal Ovary Development in the Rat : Morphologic Study and Correlation of Morphology to Neuroendocrine Parameters. *Toxicologic Pathology* **43** 343–353.

**Qi X-F, Zheng L, Lee K-J, Kim D-H, Kim C-S, Cai D-Q, Wu Z, Qin J-W, Yu Y-H and Kim S-K** (2013) HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway. *Cell Death & Disease* **28** e518.

**Rajender S, Avery K and Agarwal A** (2011) Epigenetics , spermatogenesis and male infertility. *Mutation Research / Reviews in Mutation Research* **727** 62–71.

**Rey R** (2003) Regulation of spermatogenesis. In *The Developing Testis: Physiology and Pathophysiology.*, pp 38–55. Ed O Soder.

**Rodríguez CM, Kirby JL and Hinton BT** (2002) The development of the epididymis. In *The Epididymis – from Molecules to Clinical Practice.*, pp 251–268. Eds B Robaire and BT Hinton. New York: Kluwer Academic/Plenum Publisher.

**Saitou M and Yamaji M** (2012) Primordial germ cells in mice. **4** 1–20.

**Sarraj MA and Drummond AE** (2012) Mammalian foetal ovarian development: Consequences for health and disease. *Reproduction* **143** 151–163.

**Schachter M** (2005) Chemical, pharmacokinetic and pharmacodynamic properties of statins: An update. *Fundamental and Clinical Pharmacology* **19** 117–125.

**Schagdarsurengin U and Steger K** (2016) Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. *Nature Reviews Urolology* **13** 584–595.

**Setchell BP** (1980) The functional significance of blood-testis barrier. *Journal of Andrology* **1** 3–10.

**Setchell BP and Soder O** (2003) Posnatal testicular development, cellular organization and paracrine regulation. In *The Developing Testis: Physiology and Pathophysiology. Endocrine*

*Development*, pp 24–37. Ed O Soder.

**Seth A and Sharma R** (2013) Childhood obesity. *Indian J Pediatr* **80** 309–317.

**Shaikh AA** (1971) Estrone and estradiol levels in the ovarian venous blood from rats during the estrous cycle and pregnancy. *Biology of Reproduction* **5** 297–307.

**Sheehan DM, Branham WS, Medlock KL, Olson ME and Zehr DR** (1981) Uterine responses to estradiol in the neonatal rat. *Endocrinology* **109** 76–82.

**Shrilatha B and Muralidhara** (2007) Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. *Reproductive Toxicology* **23** 578–587.

**Siavash M and Amini M** (2014) Vitamin C may have similar beneficial effects to Gemfibrozil on serum high - density lipoprotein - cholesterol in type 2 diabetic patients. *Journal of Research in Pharmacy Practice* **3** 77–82.

**Silva PV, da Silva RF, Borges C dos S, Cavariani MM, de Almeida Francia CCD, Junior FB and De Grava Kempinas W** (2016) Sexual differentiation and reproductive development of female rat offspring after paternal exposure to the anti-tumor pharmaceutical cisplatin. *Reproductive Toxicology* **60** 112–122.

**Sociedade Brasileira de Cardiologia** (2007) IV Diretriz Brasileira Sobre Dislipidemias e Prevenção da Aterosclerose. pp 1–19. Arquivos Brasileiros de Cardiologia.

**Sönmez M, Turk G and Yuce A** (2005) The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology* **63** 2063–2072.

**Sooriyaarachchi M, Narendran A and Gailer J** (2012) The effect of sodium thiosulfate on the metabolism of cis-platin in human plasma in vitro. *Metallomics* **4** 960–967.

**Stoker TE, Laws SC, Guidici DL and Cooper RL** (2000) The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. *Toxicological Sciences* **58** 50–59.

**Stuppia L, Franzago M, Ballerini P, Gatta V and Antonucci I** (2015) Epigenetics and male reproduction : the consequences of paternal lifestyle on fertility , embryo development , and children lifetime health. *Clinical Epigenetics* **7** 1–15.

**Tanaka T, Tatsuno I, Uchida D, Moroo I, Morio H, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Saito Y *et al.*** (2000) Geranylgeranyl-pyrophosphate, an isoprenoid of mevalonate cascade, is a critical compound for rat primary cultured cortical neurons to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA reductase inhibition. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* **20** 2852–2859.

**Tandon V, Bano G, Khajuria V, Parihar A and Gupta S** (2005) Pleiotropic effects of statins. *Indian Journal of Pharmacology* **37** 77–85.

**U.S. Environmental Protection Agency .** (1996) Guidelines for Reproductive toxicity risk assessment. *Environmental Protection Agency* **61** 56274–56322.

**Vaughan CJ and Gotto Jr AM** (2004) Update on Statins: 2003. *Circulation* **110** 886–892.

**Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR and Smith LB** (2008) Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *The Journal of Clinical Investigation* **118** 1479–1490.

**Westwood FR** (2008) Toxicologic Pathology The Female Rat Reproductive Cycle : A Practical Histological Guide to Staging. *Toxicologic Pathology* **36** 375–384.

**Wilson JD and Siiteri PK** (1973) Developmental pattern of testosterone synthesis in the fetal gonad of the rabbit. *Endocrinology* **92** 1182–1191.

**Yanai H and Morimoto M** (2004) Effect of ascorbate on serum lipids and urate metabolism during exhaustive training. *Clinical Science* **106** 107–109.

**You L and Sar M** (1998) Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. *Endocrine* **9** 253–261.

**Young JM and McNeilly AS** (2010) Theca: The forgotten cell of the ovarian follicle. *Reproduction* **140** 489–504.

**Zawatski W and Lee MM** (2013) Male pubertal development: Are endocrine-disrupting compounds shifting the norms? *Journal of Endocrinology* **218** 1–12.

**Zhang H, Nagaoka K, Usuda K, Nozawa K, Taya K, Yoshida M and Watanabe G** (2016) Estrogenic Compounds Impair Primordial Follicle Formation by Inhibiting the Expression of Proapoptotic Hrk in Neonatal Rat Ovary. *Biology of Reproduction* **95** 78–88.

# *Anexos*

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

# *Certificado*

Certificamos que o Protocolo nº **589-CEUA**, sobre "Exposição de ratos machos à rosuvastatina na pré-puberdade, na ausência ou presença de vitamina C:efeitos imediatos e tardios sobre o sistema genital e a fertilidade das gerações F0 e F1", sob a responsabilidade de **Wilma De Grava Kempinas**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 3 de abril de 2014.

**Prof. Dr. Wellerson Rodrigo Scarano**
Presidente da CEUA

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Campinas, 14 de junho de 2018

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